



A novel oral nutraceutical formula (PLP10) for the treatment of relapsing remitting multiple sclerosis: a randomized, double-blind, placebo-controlled proof-of-concept clinical trial

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Complete List of Authors:	Pantzaris, Marios; The Cyprus Institute of Neurology and Genetics (CING), Clinic C and PALUPA Medical Ltd Loukaides, George; The Cyprus Institute of Neurology and Genetics (CING), Neurology Clinic and PALUPA Medical Ltd Ntzani, Evangelia; University of Ioannina School of Medicine (UISM), Clinical and Molecular Epidemiology Unit, Department of Hygiene and Epidemiology Patrikios, Ioannis; The Cyprus Institute of Neurology and Genetics (CING), Clinic C and PALUPA Medical Ltd; European University Cyprus, Health Science
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1 **A novel oral nutraceutical formula (PLP10) for the**
2 **treatment of relapsing remitting multiple sclerosis: a**
3 **randomized, double-blind, placebo-controlled proof-of-**
4 **concept clinical trial**

5 **Marios C. Pantzaris, George N. Loukaides, Evangelia E. Ntzani, Ioannis S.**
6 **Patrikios**

7 The Cyprus Institute of Neurology and Genetics (CING) 1683 Nicosia, Cyprus Marios C.
8 Pantzaris Senior Consultant Neurologist Neurology Clinic C and PALUPA Medical Ltd., The
9 Cyprus Institute of Neurology and Genetics (CING) 1683 Nicosia, Cyprus George N.
10 Loukaides Clinical Dietitian/Nutritionist Neurology Clinic and PALUPA Medical Ltd.,
11 University of Ioannina School of Medicine (UISM) 45110 Ioannina, Greece Evangelia E.
12 Ntzani Assistant Professor Clinical and Molecular Epidemiology Unit, Department of
13 Hygiene and Epidemiology, PALUPA Medical Ltd at The Cyprus Institute of Neurology and
14 Genetics (CING) 1683 Nicosia, Cyprus Ioannis S. Patrikios Senior Research Scientist,
15 visiting Professor at European University Cyprus Nicosia, Cyprus, Department of Health and
16 Science. Correspondence should be addresses to e-mail: I.Patrikios@euc.ac.cy or
17 pantzari@cing.ac.cy

18
19 **Correspondence to:**

20 **Ioannis Patrikios**

21 The Cyprus Institute of Neurology and Genetics (CING)
22 Neurology Clinic C (PALUPA Medical),

1
2
3 23 6 International Airport Av.
4
5 24 P.O.Box 23462, 1683 Ayios Dometios. Nicosia, Cyprus
6
7 25 Tel: +357 22 358 600, +357 99 097 856;
8
9
10 26 i.patrikios@euc.ac.cy
11
12 27 patrikiosioannis@gmail.com
13
14
15

16 **AND**
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21 **Marios Pantzaris**
22

23
24 32 The Cyprus Institute of Neurology and Genetics (CING)
25
26 33 Neurology Clinic C (PALUPA Medical),
27
28 34 6 International Airport Av.
29
30 35 P.O.Box 23462, 1683 Ayios Dometios, Nicosia Cyprus
31
32 36 Tel: +357 22 358 600;
33
34 37 pantzari@cing.ac.cy
35
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40 medicine, randomized clinical trial.
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3 **Abstract**
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5 **Objective** To assess whether our three novel interventions, formulated based on systems
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multiple sclerosis who were either treated with disease modifying treatment or untreated.

Design 30-month randomized double-blind, placebo-controlled, parallel design, phase II proof-of-concept clinical study.

Settings Cyprus Institute of Neurology and Genetics (CING)

Participants and Interventions 80 subjects were randomized into four groups of 20. The first intervention (A) was composed of omega-3 and omega-6 polyunsaturated fatty acids at 1:1 wt/wt. Specifically, the omega-3 fatty acids were docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) at 3:1 wt/wt and the omega-6 fatty acids were linoleic acid (LA) and gamma (γ)-linolenic acid (GLA) at 2:1 wt/wt. This intervention also included minor quantities of other specific polyunsaturated, monounsaturated and specific saturated fatty acids as well as vitamin A and vitamin E (alpha-tocopherol). The third intervention (C) was γ -tocopherol alone. The second intervention (PLP10) was a combination of A and C. A fourth group of 20 received a vehicle placebo. The interventions were administered per os once daily.

Main outcome measures The primary endpoint was the annualized relapse rate (ARR) of the three interventions versus placebo at two years. The secondary end point was the time to confirmed disability progression at two years.

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3 72 **Results** The per-protocol, proof-of-concept, analysis demonstrated a 64% adjusted relative
4
5 73 reduction in ARR at two years for PLP10 versus placebo (P=0.024). Regarding the secondary
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7 74 endpoint, a relative reduction of 86% in the risk of sustained progression of disability was
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10 75 observed within the PLP10 group (p=0.047). No adverse events were reported. Interventions
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12 76 A and C showed no significant efficacy.
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23 78 **Conclusions** PLP10 treatment significantly reduced the ARR, and the risk of sustained
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25 79 disability progression without any adverse or significant side effects.
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81 **Trial registration** International Standard Randomized Controlled Trial, number
82 ISRCTN87818535.
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92 Introduction

93 Multiple sclerosis (MS) is a complex multifactorial disease that results from interplay
94 between as yet unidentified environmental factors and susceptibility genes.¹⁻³ Together, these
95 factors trigger a cascade of events, involving engagement of the immune system,
96 inflammatory injury of myelin, axons and glia, functional recovery and structural repair,
97 gliosis, and neurodegeneration.⁴ The bio-mechanisms involved are: immune-mediated
98 inflammation, oxidative stress and excitotoxicity.⁵⁻⁹ These mechanisms may all contribute to
99 oligodendrocyte and neuronal damage and even cell death, hence promoting disease
100 progression. The increasing prevalence of MS, the limited efficacy and the side effects of the
101 existing treatments urge the clinical need for the development of new, innovative, more
102 effective, safe, and preventive treatment strategies.

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104 Research has shown that multiple variables dynamically interact and many different complex
105 interrelated processes are simultaneously orchestrated for MS pathogenesis. The fundamental
106 distinctiveness of SM is not just the recognition that different specific complex factors are
107 important in disease management, but that they need to be incorporated in some meaningful
108 way to treatment selection and delivery.¹⁰ The primary challenge tackled by systems
109 scientific approach is the elucidation of how these multiple variables dynamically interact and
110 how one can apply this understanding to affect the system and achieve a desirable end.¹⁰ The
111 answer might be the simultaneous interference with all involved perturbed mechanisms, by
112 using a cocktail of different specific ingredients, potentially able through synergistic effect to
113 give a long, holistic and effective treatment (Supplementary Information Methods 1).

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115 The PUFA composition of membrane phospholipids plays a direct role in immune and non-
116 immune related inflammation. PUFA and antioxidant deficiencies along with decreased

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3 117 cellular antioxidant defense mechanisms have been reported for MS patients.¹¹ The cause of
4
5 118 these PUFA deficiencies is not entirely clear and may involve metabolic and nutritional
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7 119 alterations.¹¹
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11 121 Increased or uncontrolled inflammation contributes to several different acute and chronic
12 122 diseases and it is characterized by the production of inflammatory cytokines, arachidonic acid
13 123 (AA)-derived eicosanoids (prostaglandins (PGs), thromboxanes (TXs), leukotrienes (LTs),
14 124 and other oxidized derivatives), other inflammatory agents such as reactive oxygen species
15 125 (ROS), nitric oxide (NO), and adhesion molecules (Fig 1).¹² During inflammation glutamate
16 126 homeostasis is altered by activated immune cells releasing increased quantities of glutamate
17 127 that can result in over activation of glutamate receptors and in return excitotoxic
18 128 oligodendroglial death.^{7,13} As such, among others, membrane-related pathology, immune-
19 129 mediated inflammation, oxidative stress and excitotoxicity provide potentially useful
20 130 combined targets for intervention in MS.
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36 132 *In vitro and in vivo* studies have demonstrated that dietary EPA, DHA, LA, and GLA can be
37 133 implicated and modulate almost all known complex network of events and pathways
38 134 repertoire in MS pathophysiology. Brain membrane fatty acid composition can be modified
39 135 with dietary supplementation, but the process has been showed to be age dependent (it takes
40 136 much longer in adults vs. developing brains) as well as possibly dependent on the quantities
41 137 of the dietary/supplemented PUFAs.¹⁴ Both human and animal studies proved that diets high
42 138 in DHA and EPA increase the proportion of these PUFA in the membranes of inflammatory
43 139 cells and reduce the levels of AA.^{12,15} The anti-inflammatory properties of omega-3 include
44 140 production of PGs and TXs of the 3-series and LTs of the 5-series (Fig 1).^{14,16} Resolvins and
45 141 protectins are biosynthesized from omega-3 fatty acids via cyclooxygenase-2/lipoxygenase
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3 142 (COX-2/LOX) pathways and they promote control of inflammation in neural tissues (Fig
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5 143 1).¹⁷⁻²¹ T-cell proliferation in acute and chronic inflammation can be reduced by
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7 144 supplementation with either omega-6 or omega-3 PUFA.²² Furthermore, vitamin E is an
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9 145 important antioxidant that can interrupt the propagation of free radical chain reactions.²³
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11 146 Vitamin E (alpha-tocopherol, an isoform of vitamin E) efficiently detoxifies hydroxyl,
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13 147 perhydroxyl and superoxide free radicals.²⁴ However γ -tocopherol (another isoform of
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15 148 vitamin E) seems to be more efficiently implicated in trapping NO radicals.²⁵ In addition
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17 149 alpha-tocopherol exerts non-antioxidant properties, including modulation of cell signaling
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19 150 and immune function, regulation of transcription, and induction of apoptosis.²⁶
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23 152 Moreover, omega-3 fatty acids electrophilic derivatives formed by COX-2, in activated
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25 153 macrophages can stimulate the nuclear respiratory factor (Nrf2) inducing the transcription of
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27 154 neuroprotective and antioxidant related genes, and can activate the peroxisome proliferator-
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29 155 activated receptor (PPAR) γ for anti-inflammatory response.²⁷⁻²⁹ In animal studies, EPA and
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31 156 DHA proved to be endogenous ligands of RXRs, with positive effect on neurogenesis.³⁰
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33 157 Additionally, in 2008 Salvati and coworkers reported evidense of accelerated myelination in
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35 158 DHA- and EPA-treated animals.³² Moreover, DHA and EPA are reported to significantly
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37 159 decrease the levels of metalloproteinases (MMP) -2, -3, -9, and -13 with a significant role in
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39 160 the migration of lymphocytes into the CNS by inducing disruption of the blood brain barrier
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41 161 (BBB), an important step in the formation of MS lesions.³³⁻³⁹
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45 163 Based on the above, specific PUFA and antioxidant vitamins fulfill the criterion of biologic
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47 164 plausibility and have the potential to diminish MS symptoms severity and activity, even
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49 165 promoting recovery (remyelination).¹¹ Overall, PLP10 includes multiple ingredients
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3 166 interacting with key interconnected components within functional network modules, each
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5 167 contributing a fraction of the effects of perturbations that cause the disease.⁴⁰
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10 169 In our phase II, single-center, randomized, double-blind, placebo-controlled, proof-of-
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12 170 concept clinical trial we intended to evaluate the therapeutic ability of PLP10 and of two
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14 171 other interventions (A and C) consisting of PLP10 constituent partial fractions versus
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16 172 placebo, when used on RRMS patients.
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22 23 175 **Methods**

24 25 26 176 **Patients**

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28 177 Eligibility criteria were an age of 18 to 65; a diagnosis of RRMS according to the McDonald
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30 178 criteria; a score of 0.0 to 5.5 on the EDSS, a rating that ranges from 0 to 10, with higher
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32 179 scores indicating more severe disability; MRI showing lesions consistent with MS; and at
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34 180 least one documented clinical relapse either receiving or not disease modifying treatment
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36 181 (DMT) within the 24 months period before beginning (enrollment) of the study. Patients were
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38 182 excluded because of a recent (<30 days) relapse, prior immunosuppressant or monoclonal
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40 183 antibodies therapy, pregnancy or nursing, other severe disease compromising organ function,
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42 184 progressive MS, history of recent drug or alcohol abuse, use of any additional food
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44 185 supplement, vitamin, or any form of PUFA, history of severe allergic or anaphylactic
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46 186 reactions or known specific nutritional hypersensitivity. No monitor or limitations on
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48 187 patients' daily diet habits were included in the study design since the quantities of the
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50 188 ingredients within the formulas daily-dosage could not be significantly affected or spoiled by
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52 189 any confounding factors within any known global daily food diet (see procedures, treatment
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54 190 regimen and end-points).
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192 The study was conducted in accordance with the standards of the International Conference of
193 Harmonization Guidelines for Good Clinical Practice. The protocol was developed by the
194 investigators and it was approved by the Cyprus National Bioethics Committee and was
195 overseen by an independent safety-monitoring committee evaluating the safety and over-all
196 benefit-risk profiles. The adherence of care providers with the protocol was assessed by an
197 external committee assigned by the funder of the project through reviews of case report
198 forms. All patients gave written informed consent at the time of enrolment.

199

200 **Randomisation and masking**

201 Patients were randomly assigned to four intervention groups in a 1:1:1:1 ratio stratified by
202 gender (women to men, 3:1). Randomization was facilitated by a lottery-type pool of
203 numbered balls. Patients were randomly assigned to treatment in blocks of four by flipping a
204 coin as follows: for the first two drawn balls heads stratified them to the groups A/B and tails
205 stratified them to the groups C/D. The other two balls were stratified accordingly. A second
206 toss of the coin assigned the two patients to group A (head)/B (tail) or group C (head)/D
207 (tail). The randomization scheme was generated, performed and securely stored by Helix
208 Incubator Organization of Nicosia University (HIONU).

209

210 The interventions had identical appearance and smell in dark bottles (15 daily-dose
211 portions/bottle) under nitrogen bed and labeled by HIONU with code numbers, unidentifiable
212 for both patients and investigators. Study data were collected by the investigators and saved
213 by the HIONU that also held the blinded codes of the study. All study personnel involved in
214 the conduct of the study were blinded throughout the study. Treating/examining physician,

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3 215 other investigators, pharmacist, neuroradiologist and patients were masked to treatment
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5 216 allocation.

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10 218 **Procedures and end points**

11 219 The specific omega-3 (re-esterified glycerides) and omega-6 (glycerides) raw materials were
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13 220 purchased according to the required interventions' PUFA-fraction specification (molecular
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15 221 structure, quantity/ratio and quality) with vitamin E (alpha-tocopherol) used as antioxidant
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17 222 stabilizer by the supplier. The vitamins and masking aroma were purchased separately. The
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19 223 mixing of fractions to the final required intervention-composition specification was always
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21 224 performed by the same team of scientists under the supervision of the involved medical
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23 225 biochemist and lipidology specialist, under appropriate conditions every six months.

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25 226 Interventions were stored refrigerated in dark until use. See Supplementary Information
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27 227 Methods 1 and 2 for intervention specification detailed description and study/intervention
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29 228 rational.

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35 230 Participants were randomly assigned to receive a daily dose of a mixture of EPA (1,650mg) /
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37 231 DHA (4,650mg) / GLA (2,000mg) / LA (3,850mg) / total other omega-3 (600mg) / total
38
39 232 MUFA (1,700mg) + total SFA (18:0 160mg, 16:0 650mg) / vitamin A (0.6mg) / vitamin E
40
41 233 (22mg) (intervention A, group A); or composed mixture of pure γ -tocopherol (760mg)
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43 234 dispersed in pure virgin olive oil (16,137 mg) as delivery vehicle (intervention C, group C);
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45 235 or a mixture of intervention formula A with intervention C without the pure virgin olive oil
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47 236 (intervention B, named PLP10, group B); or placebo composed of pure virgin olive oil
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49 237 (16,930mg) (intervention D, group D). Citrus-aroma was used as masking agent of the taste
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51 238 and odor and added in each one of the intervention for a total of 19.5ml dosage of solution
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53 239 per day. The institution's pharmacist was responsible for the appropriate storage and handling
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3 240 of the interventions to the individual participants. The interventions were taken orally once
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5 241 daily 30 minutes before dinner by a dosage calibrated cup for 30 months. The ingredients,
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7 242 ratio and dose have been selected based on their biophysical interrelation to the total known
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9 243 multiple MS causing factors, their biochemical importance and the role expected to play in
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11 244 the normalisation and treatment of the involved complex network of events in the disease
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13 245 pathophysiology. Moreover, the high intake dosage was used to overcome any abnormal
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15 246 dietary accumulation of related agents as a result of patients' food intake habits, irrespective
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17 247 of geographical origin, in relation to the daily consumption ratio of the total fatty acid intake;
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19 248 in order to end-up with omega-3 to omega-6 PUFA indicated physiological body ratio
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21 249 composition of 1:1 wt/wt.
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27 251 The period beginning from July 1st 2007 (enrolment) until December 31st 2007 (entry
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29 252 baseline) was used for normalization period. This six-month normalization period would
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31 253 allow the interventions' agents to exert their beneficial effect (for the
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33 254 incorporation/normalization of cell membranes by oral PUFA, since they need four to six
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35 255 months to exert pivotal action on immune and neural cells, correction of antioxidant
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37 256 deficiency and body PUFA, and optimal normalization of EPA and DHA levels/ratio).⁴¹⁻⁴³
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39 257 The study was completed on December 31st 2009 and the recording of relapses continued
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41 258 until December 31st 2010.
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47 260 Depending on their clinical status and in accordance with the ethical issues governing clinical
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49 261 trials participants continued receiving the indicative regular available treatments, according to
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51 262 international guidelines with persistent evaluation of any side-effects and adverse events.
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53 263 The study was designed to end 30 months after enrolment and clinical assessments were
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55 264 scheduled at entry baseline, 3, 9, 15, 21 and 24 months on-treatment. Patients were also
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3 265 clinically examined by the treating neurologist within 48 hours after the onset of new or
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5 266 recurrent neurologic symptoms.
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9 268 The primary end point was the ARR at two years. A relapse was defined as new or recurrent
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11 269 neurologic symptoms not associated with fever or infection that lasted for at least 24 hours
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13 270 and accompanied by new neurologic signs. Relapses were treated with methyl-prednisolone
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15 271 at a dose of 1g intravenous per day, for three days followed by prednisone orally at a dose of
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17 272 1mg/kg of weight per day on a tapering scheme for three weeks. The secondary end point at
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19 273 two years was the time to confirmed disability progression, defined as an increase of 1.0 or
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21 274 more on EDSS, confirmed after six months (progression could not be confirmed during a
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23 275 relapse). The final EDSS score was confirmed six months after the end of the study. A post-
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25 276 hoc analysis was performed assessing the proportion of patients free from new or enlarging
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27 277 T2 lesions on brain MRI scans at the end of the study for the per-protocol participants of the
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29 278 group receiving the highest effective intervention versus placebo. Comparison was made only
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31 279 versus the available archival MRI scans up to three months before the enrolment date. MRI
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33 280 scans were performed and blindly analyzed at an MRI evaluation centre. The patients
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35 281 continued to be followed for additional 12 months after completion of the trial and relapses
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37 282 were recorded. Finally, patients were strongly encouraged to remain in the study for follow-
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39 283 up assessments even if they had discontinued the study drug.
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47 285 Blood samples were collected from all randomized patients at the time of enrolment, at every
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49 286 scheduled clinical assessment and during relapses. To check individual compliance with
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51 287 intake, the fatty acids composition of patients' red blood cells' membranes was determined,
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53 288 by gas chromatography, according to a standard protocol. The fatty acid analyses were
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55 289 performed after study termination and thus did not influence the blinding.
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291 The involved neurologist was experienced with more than 20 years in practice and trained to
292 standardise EDSS scoring procedures, examined patients, made all medical decisions,
293 determined the EDSS score and reviewed adverse or side-effects. The medical biochemist,
294 specialist on lipidology and immunology and the registered clinical dietitian, members of the
295 investigator team were experienced with more than 25 years in practice. Patients were able to
296 contact the neurologist at any time if there was any adverse event, side-effect or allergic
297 reaction. The study drug was not suspected to have any clinical or laboratory adverse effects
298 different from placebo that could disturb the double-blind nature of the trial. Therefore, the
299 same study-neurologist functioned as both the treating and evaluating physician.

300

301 Safety measures were assessed from the time of enrollment until 12 months following study
302 completion. Haematological and biochemical tests were performed at enrolment and at every
303 12 months, including full blood count, renal and liver function tests, proteins, cholesterol,
304 triglycerides, glucose and electrolytes.

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306 The whole procedure followed the clinical trial guidelines as required by the USA Food and
307 Drug Administration, European Medicines Agency, and the Committee for Medicinal
308 Products for Human Use.⁴⁴

309

310 **Statistical analysis**

311 Power calculations could not be done before the study because of the lack of information
312 from previous studies on potential effect sizes. Based on the population size of our country
313 and the centre of reference, the CING, we were able to enrol the 20% of the total RRMS

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3 314 patients eligible for treatment in the trial. Sample size was strictly based on this subjects'
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5 315 availability parameter and the novelty of the assessed intervention.
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9 317 Baseline characteristics were compared across all intervention groups by ANOVA or
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11 318 Kruskal-Wallis rank test for continuous variables and by an exact chi-squared test for
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13 319 categorical variables, as appropriate.
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18 321 For the primary outcome, the ARR was analysed in a pair-wise fashion for the active
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20 322 interventions compared to placebo using negative binomial regression models adjusted for
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22 323 number of relapses within two years before baseline, EDSS score at baseline and DMT. The
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24 324 relapse rate was calculated as the total number of relapses divided by the total number of
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26 325 patient-years followed for each treatment group. ARR differences were also calculated
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28 326 among all comparable parameters and reported as percent difference.
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33 328 For the secondary end-point outcome, the time to disability progression, Kaplan–Meier
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35 329 curves were constructed. Progression to disability and time thereof was compared in a pair-
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37 330 wise fashion for the active interventions versus placebo by the log-rank test in the main
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39 331 analysis and by Cox proportional-hazards models with adjustment for baseline EDSS score,
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41 332 age and DMT in the supportive analysis. Each test was performed with a significance level of
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43 333 0.05. Multivariate models considered all variables with $P < 0.1$ on univariate models. There
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45 334 was no overt violation of the proportionality assumption.
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51 336 Both, per-protocol and intention to treat (ITT) analyses were performed, for different sets of
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53 337 research questions to be answered, and both are reported. Missing data of the five lost to
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55 338 follow patients were imputed by use of the last-observation-carried-forward (LOCF)
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3 339 approach. Due to the proof-of-concept design of the study, the considerable non-adherence
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5 340 rate (49%) and the interpretation issues caused thereof regarding the ITT analysis, the per-
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7 341 protocol analysis considered being more informative and appropriate method approach to
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10 342 answer the research addressed questions of efficacy of the interventions when subjects were
11
12 343 continuously following the protocol. All analyses were performed with STATA SE 10.0
13
14 344 (College Station, TX, USA). P-values are two-tailed.

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17 18 346 **Role of the funding source**

19
20 347 The funders had no role in study design, data collection and analysis, decision to publish, or
21
22 348 preparation of the manuscript. All members of the writing group had full access to all study
23
24 349 data and contributed to its interpretation and prepared, reviewed, and approved the
25
26 350 manuscript for submission. All authors had final responsibility for the decision to submit the
27
28 351 paper for publication.
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33 34 353 **Results**

35 36 354 **Study population**

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38 355 From July 2007 through December 2010 (including the 12-month extended period), a total of
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40 356 80 MS patients were randomly assigned to a study group at the CING (tertiary neurological
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42 357 center).
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48 359 Among the 80 patients, 20 patients were randomly assigned to each of the three groups to
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50 360 receive the interventions and 20 to receive placebo (Fig 2). Baseline characteristics of both
51
52 361 the ITT and the per-protocol populations were similar across groups (Table 1A and 1B).
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54 362 Total drop-out patients completed follow-up until study completion and were included in the
55
56 363 ITT analyses (Table 3). Five patients were totally lost to follow before their first scheduled
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3 364 visit and two patients dropped-out before their first scheduled visit progressed to secondary
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5 365 progressive MS. Fifteen patients dropped-out without successfully completing the
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7 366 “normalization” period including five pregnancies. Another 17 patients dropped-out early
8
9 367 after entry baseline. Seven patients that dropped out were given monoclonal antibody
10
11 368 treatment (natalizumab). Overall, a total of 41 (51%) patients completed the 42-month study
12
13 369 (July 2007 through December 31st 2010, including the 12-month extended period) where one
14
15 370 patient from group A and two from the placebo group transferred on natalizumab, and 39
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17 371 (49%) patients either withdrew (drop-out) or lost to follow. Reasons for study-interventions
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19 372 discontinuation are listed in Figure 2.
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374 **Efficacy**

375 **Relapses**

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29 376 As a proof-of-concept trial we primarily needed to answer whether the interventions were
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31 377 effective for those MS patients who adhere to the assigned treatment, the per-protocol
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33 378 analysis.⁴⁵ For the sake of methodological comprehensiveness we also present the ITT
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35 379 analysis as a secondary analysis, to answer a different question, complementary to our core
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37 380 hypothesis; like what happened to MS patients who were placed on the interventions (the
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39 381 effect of assignment).⁴⁵ Otherwise, as a result of a high drop-out rate, an ITT analysis will not
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41 382 likely be able to show the superiority of an intervention even if it is effective.⁴⁵ In any
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43 383 instance, the proper approach of evaluating a study data is to understand what question
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45 384 prompted the research and assure that the analysis is appropriate for providing the answer
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47 385 whatever it is called. Both analyses can be performed for a study, using the results from the
48
49 386 different analyses to answer different research questions.⁴⁵ These interventions are original,
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51 387 composed by a different treatment rational, the SM, never tested before and the important
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53 388 main concern was to evaluate their efficacy and safety based on the per-protocol treated MS
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3 389 patients, without any peripheral noise. The question that had to be answered was: “what
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5 390 happens to the patients that are placed and stick on the specific treatment”.
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9 392 Regarding the per-protocol analysis, during the first year of treatment, the ARR was 0.80,
10
11 393 0.40, 0.78 and 0.83 for the four intervention groups respectively. During the second year, the
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13 394 ARR was 0.90, 0.40, 0.67 and 1.25 for the four intervention groups respectively. Overall, for
14
15 395 the two-year primary end point, 8 relapses were recorded for the 10 patients in PLP10 group
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17 396 (0.40 ARR) versus 25 relapses for the 12 patients in placebo (1.04 ARR), a 64% adjusted
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19 397 relative rate reduction (RRR) for the PLP10 group (RRR 0.36, 95% confidence interval (CI)
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21 398 0.15 to 0.87, $p=0.024$) (Tables 2A, 4 and Fig 3A and 3C). Excluding patients on monoclonal
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23 399 antibody (natalizumab) treatment, the observed adjusted RRR became stronger (72%) over
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25 400 the two years (RRR 0.28, 95% CI 0.10 to 0.79, $p=0.016$, Tables 2B and 4). Pair-wise
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27 401 comparisons for the other two groups against placebo did not yield statistically significant
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29 402 results (Tables 2A, 2B). The proportion of patients with ≤ 1 relapse for the two years on-study
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31 403 was higher in the PLP10 group than in the placebo group (90% vs. 42%, $p=0.030$, Table 4).
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33 404 Seeking to investigate further the observed difference, we compared the relapse rate during
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35 405 the 24 months before entry to the study to the 24 months on-treatment for each intervention
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37 406 group. We observed a statistically significant relative reduction in the ARR (70%) only in the
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39 407 PLP10 group (RRR 0.30; 95% CI 0.14 to 0.65, $p=0.003$, Table 2A); within-group
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41 408 comparisons for the three other groups ARR reduction was not significant and remained not
42
43 409 significant when natalizumab treated patients were further excluded from the analysis. The
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45 410 effect of PLP10 through time at different time-windows versus placebo for all-time on-study
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47 411 patients is shown in Figures 3A to 3D. The ARR analysis, within time-windows, was not an
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49 412 assigned endpoint, but it could help in the process of evaluating parallel information as the
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51 413 time needed for a specific treatment intervention activity to be evident, as well as the efficacy
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3 414 profile through time. PLP10 reached maximum effect within a year on-treatment (counting
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5 415 from the entry baseline) and remained stable at an ARR of 0.4, displaying a steadily reduced
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7 416 ARR with long free-relapse time-windows. These group B characteristics are considered
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9 417 important parameters of a successful MS treatment where the rule than the exception is the
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11 418 heterogeneity among patients' disease evolution. Specifically, Figure 3D demonstrates the
12
13 419 dispersion of relapses throughout the 2-year period of all-time on-study (excluding patients
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15 420 on natalizumab) of PLP10 (n=10) vs. placebo (n=10). Placebo, in line with the existing
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17 421 knowledge of how relapse history works in relation to future relapses on MS patients
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19 422 (contagion phenomenon) indicates the expected linearly increased trend of the relapse
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21 423 incidence.⁴⁶ The same phenomenon was true for the groups A and C. Finally, during the 12
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23 424 month post-study extended period (January 1st 2010 to December 31st 2010) all-time on-study
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25 425 patients that received PLP10, showed persistent benefit in the ARR compared to placebo (six
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27 426 relapses for the 10 subjects within PLP10, 0.6 ARR vs. 19 for the 12 subjects within placebo
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29 427 group, 1.58 ARR) indicating a statistically significant 62% adjusted relative rate reduction in
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31 428 the ARR for the PLP10 group (RRR 0.38, 95% CI 0.12 to 0.99, p=0.046).
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430 Regarding the ITT analysis, within PLP10 group, none of the nine drop-out patients changed
431 to natalizumab and two out of nine discontinued because of pregnancy, whereas two out of
432 seven drop-out patients from the placebo group changed to natalizumab (a total of four
433 patients within the placebo arm population were on natalizumab, including the two patients
434 that transferred while all-time on-study versus none within PLP10 group (Supplementary
435 Information Fig 1). As reported, natalizumab reduces ARR by 68%, decreases the possibility
436 of disability progression by 43% with 57% of patients free of new or enlarging T2 lesions on
437 MRI scans compared to 15% on placebo.⁴⁷ The relapses of the drop-out patients are reported
438 in Table 3A. As expected no statistically significant differences in the ARR were calculated

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3 439 for the comparison of any group versus placebo for the 24 months on-treatment, with a 0.75
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5 440 ARR within PLP10 (30 relapses) and 1.03 ARR within placebo (41 relapses) group, a 27%
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7 441 ARR reduction (Table 3B). Interestingly, despite the high non-adherence rate, there was a
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9 442 statistically significant difference for the comparison of the ARR in the 24 months before
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11 443 entry baseline to the 24 months on-treatment for the PLP10 group (RRR 0.45, 95% CI 0.26 to
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13 444 0.78, $p=0.005$).

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446 **Disability progression**

447 Regarding the per-protocol analysis, at two years, the time to disability progression, with
448 confirmation after six months (secondary end-point) was significantly longer only with
449 PLP10. The cumulative probability of disability progression was 10% in the PLP10 group
450 and 58% in the placebo group ($p=0.019$) (Supplementary Information Fig 2). After excluding
451 patients on natalizumab, there was an increased statistically significant difference between
452 the PLP10 and the placebo group for the same analysis ($p=0.006$) (Fig 4A). At two years, the
453 cumulative probability of progression was 10% in the PLP10 group and 70% in the placebo
454 group, which represents a decrease of 60 percentage points or a relative 86% decrease in the
455 risk of sustained progression of disability within PLP10 group (adjusted hazard ratio, 0.11;
456 95% CI 0.01 to 0.97, $p=0.047$). One versus seven out of ten patients progressed to confirmed
457 disability in the PLP10 and the placebo groups respectively when patients on natalizumab
458 were excluded. No statistically significant difference was observed for any comparison of the
459 other two groups compared to placebo (Fig 4A and Supplementary Information Fig 2).

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461 Regarding the ITT analysis, at two years, the cumulative probability of progression was 10%
462 in the PLP10 group and 35% in the placebo group ($p=0.052$, a trend for an effect), which
463 represents a decrease of 25 percentage points or a relative 71% decrease of the PLP10 for the

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3 464 risk of sustained progression of disability (adjusted hazard ratio 0.22, 95% CI 0.04 to 1.07,
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5 465 $p=0.06$) (Fig 4B). Two versus seven out of the total randomized patients progressed to
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7 466 confirmed disability in the PLP10 and the placebo groups respectively. No significant
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10 467 differences were observed for groups A or C against placebo (Fig 4B). The mean change in
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12 468 Expanded Disability Status Scale (EDSS) score as a function of visit number is shown in
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14 469 Figure 5.

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18 471 **MRI**

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20 472 Over two years, the MRI results support the overall conclusion from the study that PLP10 has
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22 473 a positive effect on disease activity since only 29% from the PLP10 group as opposed to 67%
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24 474 from the placebo group developed new or enlarging T2 lesions (57% relative risk reduction).
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26 475 Excluding patients on natalizumab there is an increased relative risk reduction (64%) between
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28 476 PLP10 as opposed to placebo with 29% of patients on PLP10 and 80% on placebo with
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30 477 development of new or enlarging T2 lesions (Table 4).
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35 479 **Safety**

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37 480 Over the course of the 30 month study no significant adverse events were reported from any
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39 481 group. According to a questioner procedure the only aetiology for drop-outs was the
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41 482 palatability and smell of the formula preparations. Nausea was reported by two patients. No
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43 483 abnormal values observed on any of the biochemical and haematological blood tests. No
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45 484 allergic reactions reported.
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50 486 **Discussion**

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52 487 In this proof-of-concept clinical trial assessing the safety and efficacy of three cocktail
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54 488 intervention formulas in RRMS, we observed a significant benefit for the novel PLP10
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3 489 intervention compared to placebo for both the ARR and the progression to disability. Our
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5 490 results include analyses pertaining to a total of 42 months study collected data, including the
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7 491 12-month, free of intervention treatment, extension period. We focused on the per-protocol
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9 492 data analysis since it is the appropriate method to best provide the answer to the proof-of-
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11 493 concept trial-addressed question. The high drop-out rate was solely the result of formulas
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13 494 palatability, a common phenomenon in trials using oily interventions. We thus present our
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15 495 main per-protocol analysis, as well as a subgroup analysis excluding patients on natalizumab.
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17 496 We have found a statistically significant reduction in the ARR and the disability progression
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19 497 comparing not only patients on PLP10 versus placebo but also comparing the ARR of the
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21 498 PLP10 patients in the 24-month period prior to the study to the ARR of the 24 months on-
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23 499 study; the observed differences became larger when patients that received natalizumab (the
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25 500 most potent disease modifier) were excluded. The ARR decreased within a year on PLP10
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27 501 and significantly remained stable until study completion. Statistically significant difference of
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29 502 ARR between patients on PLP10 versus placebo continued for the additional 12 month
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31 503 extended period (persistent effect) without significant difference on DMT. These clinical
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33 504 findings are supported by the results regarding the MRI analysis where the proportion of
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35 505 patients free from new or enlarging brain T2 lesions was also higher in PLP10 group versus
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37 506 placebo. The persistent effect within the extended period it is considered of major importance
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39 507 and supportive of the results since it is in agreement with the very long washouts, reported
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41 508 necessary, for omega-3 fatty acids and especially DHA to return towards pretreatment values
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43 509 within the fatty acids of plasma, platelets, monocytes and red blood cells.⁴² This study also
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45 510 provides important 30-month, placebo-controlled information about the safety of PLP10, A
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47 511 and C interventions, where no any severe or significant side-effects have been reported.
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3 513 As medications used to treat MS become increasingly highly specific and potent, attention to
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5 514 safety is paramount. Current available treatments are products of reductionism, partially
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7 515 effective, associated with severe side effects without (re)myelinating or neuroprotective
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9 516 abilities. Interferons and glatiramer acetate, the most widely used first-line MS drugs
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11 517 available today, are associated with the least severe side-effects among MS therapies but they
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13 518 are reported with only 29-33% ARR reduction and with no significant effects on the
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15 519 progression of disability. Natalizumab as previously discussed and Fingolimod with 54%
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17 520 ARR reduction (without significant benefit on the progression of disability) are second-line
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19 521 drugs associated with severe side-effects.^{47, 48}
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25 523 No existing MS treatment has ever been designed as a result of SM concept approach or with
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27 524 a potential to effectively stimulate intrinsic remyelinating and neuroprotecting mechanisms or
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29 525 exert such an action. Now we propose that a holistic SM model approach has to be applied by
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31 526 synchronized action on all involved perturbed mechanisms. PLP10 has innovative
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33 527 characteristics like no any other intervention or medication tried before for MS treatment,
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35 528 with unique efficacy abilities through different mechanisms of action, probably by the
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37 529 synergistic effect of its constituent ingredients. PLP10 has all the characteristics of a medical
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39 530 food with the action to feed a normal metabolic process by supplying nutritional structural
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41 531 membrane precursors, building blocks, and vitamins from dietary sources that enhance
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43 532 remyelination and neuroprotection and simultaneously promote normalization of all cellular
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45 533 membranes lipid content. The intention is to normalize the specific nutritional requirements
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47 534 of the MS patients.
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54 536 Different factors and molecular entities appear to be part of the possible aetiology for MS
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56 537 with specific PUFA and antioxidants found to be key substances related to all known
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3 538 pathogenic and recovery mechanisms. But, it is well established that MS patients are
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5 539 characterized with significant deficiencies in antioxidant efficiency as well as in PUFA levels
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7 540 in blood and cellular membranes.^{11, 49-51}
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11 542 According to one hypothesis, the change in the ratio of omega-3 to omega-6, due to
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13 543 increasing consumption of omega-6 PUFA, meaning high accumulation of AA, in the
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15 544 Western diet, may be one of the major factors responsible for the increasing incidence of
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17 545 inflammatory diseases relative to populations. In the Western diet, the ratio of omega-3 to
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19 546 omega-6 is about 1:20–30; in populations that consume fish-based diets, the ratio is about
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21 547 1:1–2.^{52, 53} The intervention daily dose was aiming and believed to be high enough to
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23 548 restore/amplify body efficient antioxidant activity and ensure cellular membranes lipid profile
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25 549 normalization (PUFA content) and simultaneously potentiate involvement of the ingredients
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27 550 in the anti-inflammatory and recovery mechanisms. Diet fatty acid molecules need about six
28
29 551 months period to exert their beneficial effect and this essential parameter was for the first
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31 552 time under consideration in our study design.⁴² This chronotherapy parameter it is of major
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33 553 importance in line with the SM treatment philosophy and if it is not included in the trial
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35 554 design the possibility of misleading result evaluation greatly increases. In fact, considering
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37 555 that omega-3 supplementation can release and replace excess AA within the cellular
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39 556 membranes, we can speculate that an increased inflammatory activity can possibly result
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41 557 during the first six months of supplementation (during normalization period).
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49 559 The maintenance of myelin requires continued turnover of its components throughout life.^{54,55}

50 560 In addition to the EPA, DHA, LA, and GLA, PLP10 was containing limited quantities of
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52 561 other structural PUFA, specific MUFA (mostly oleic acid) and SFA (palmitic and stearic
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54 562 acid), specifically to provide a direct source for neuronal cell membranes rehabilitation and
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3 563 for (re)myelination and neuroprotection since they are all major components, precursors and
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5 564 building blocks of any new physiological myelin and cellular membranes in general.

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7 565 Assembly of the correct molecules into myelin membrane may be especially critical during
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10 566 active synthesis. Possibly, if critical constituents aren't available or are metabolically
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12 567 blocked, amyelination, dysmyelination or demyelination may ensue.⁵⁶

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16 569 The well known and established safety of the ingredients used and the protocol guidelines
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18 570 were supportive reasons for us to proceed with the clinical study even though with limitation
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20 571 on the pre-estimation of required trial sample size as it was discussed in method section. The
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22 572 adherence of the subjects is another issue but the duration of the study (42 months) is adding
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24 573 power to the results;⁴⁴ having the research questions been consciously and carefully
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26 574 approached and answered. Furthermore, the statistical methodologies used along with the
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28 575 appropriate adjustments, broadly accepted for MS clinical trials, power the findings, results,
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30 576 and significance. The baseline characteristics of the treatment arms could possibly be
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32 577 considered indicative of four very active groups of patients but that was the result of the
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34 578 limited number of RRMS population eligible for the study within Cyprus. On the other hand
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36 579 the balanced baseline characteristics without statistical differences, the statistical adjustments
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38 580 (for all important baseline parameters i.e. relapses, EDSS, age and DMT) and the
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40 581 randomization within four different groups are the safety valves against data
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42 582 misinterpretation. It is possible to question why DMTs efficacy cannot be emerged out of the
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44 583 data analysis, of the four treatment arms, and in accordance to their published values. We
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46 584 believe that the limited efficacy of the DMTs, the sample size and the statistical adjustments
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48 585 were strong limiting determining factors for such an indication to be countable. An additional
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50 586 argument is that the efficacy reported for the analysis of pre-treatment (24 months before
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52 587 entry baseline) vs on-trial ARR could be considered as potentially biased due to differences
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3 588 of how relapses were defined during the course of a study compared to pre-treatment period;
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5 589 or due to regression to the mean or placebo effect. This analysis was performed as an
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7 590 additional exploratory analysis that we were able to do due to the availability of data. The
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9 591 relapses of the two pre-treatment years were drawn out of the patients' archival records by
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11 592 the same treating neurology involved in the study (MP), and according to the patients'
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13 593 hospitalization date for receiving intravenous methyl-prednisolone. This analysis was not
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15 594 used as a primary or a secondary end-point under investigation although it is usually reported
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17 595 by many clinical studies. As a matter of fact many early phase trials are based only on such
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19 596 an analysis (before vs after treatment results). In almost all MS trials the number of relapses
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21 597 within the two years before baseline is a factor under adjustment for the statistical analyses.⁴⁸
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23 598 The inclusion of the post-hoc MRI analysis is another limiting factor that needs attention
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25 599 since it was used as an additional aside exploratory approach (due to study budget limitations
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27 600 it was not possible to be used as a formal endpoint); but the MRI evaluation was blinded and
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29 601 can be considered as representative of the randomized subjects within the treatment arms. As
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31 602 far as the regression to the mean and the placebo effect concerns we believe that the 6-month
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33 603 normalization period is an accountable and valuable eliminating factor of the possible effect;
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35 604 as well as the presence of four groups, where only the PLP10 treatment arm is associated
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37 605 with statistically significant efficacy vs placebo. It is a placebo-controlled study after all.
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45 607 Our observations are consistent with the idea that simultaneous availability of specific PUFA
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47 608 along with other major membrane and myelin building blocks in combination with specific
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49 609 antioxidants, within optimum quantity, quality, ratio and structural form can possibly result to
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51 610 a more appropriate holistic therapy reducing MS disease activity. This is probably succeeded
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53 611 through synergistic and/or simultaneous effect on the interactions and dynamics of the most
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55 612 probable environmental and biological disease causing factors that induce complex biological
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3 613 network of events for disease pathogenesis and evolution; as well as on the protective and
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5 614 reparative mechanisms. We can additionally speculate that the nature of the intervention
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7 615 formula cannot be prohibitive for its use as preventive regimen and does not preclude
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9 616 probable positive efficacy on the other types of MS, but has to be further investigated. A
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11 617 larger size multicenter clinical trial will better establish PLP10 place in the armamentarium of
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13 618 treatments for MS.
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620 It is commonly accepted that nutrition is one of the possible environmental factors involved
621 in the pathogenesis of MS, but its role as complementary MS treatment is unclear and largely
622 disregarded.⁵⁷ Dietary antioxidants and fatty acids may influence the disease process in MS
623 by reducing immune-mediated inflammation, oxidative stress and excitotoxic damage.¹¹
624 Present data reveal that healthy dietary molecules have a pleiotropic role and are able to
625 change cell metabolism from anabolism to catabolism and down-regulate inflammation by
626 interacting with enzymes, nuclear receptors and transcriptional factors.⁵⁷ The present study,
627 for the first time provides strong link evidence between dietary, metabolic, immunological,
628 and neurobiological aspects of MS after three quarters of a century of unsuccessful scientific
629 efforts. This might probably be the beginning of opening new horizons and new avenues in
630 the approach of MS prevention and treatment, and possibly of other multifactorial chronic
631 diseases, including neurodegenerative and autoimmune as well.
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1a.					
Characteristics	Group A (n=20)	Group B† (n=20)	Group C (n=20)	Placebo (n=20)	P- value
Sex					
Female - no. (%)	15 (75)	15 (75)	15 (75)	15 (75)	1.000
Age (yr)					
Mean ± Standard Deviation (SD)	38.0±11.9	36.9±8.4	37.7±8.7	38.1±10.9	0.982
Median (Range)	38.0 (22–65)	37.0 (25–61)	36.5 (24–54)	36.0 (21–58)	
Treatment history					
Patients on DMT- no. (%)	11 (55)	9 (45)	12 (60)	10 (50)	0.875
Pre-treatment disease duration (yr)					
Mean ± SD	9.0±7.6	8.6±4.8	8.6±5.3	7.7±5.7	0.909
Median (Range)	7.5 (2–37)	8.0 (2–20)	8.0 (3–24)	6.5 (2–25)	
Pre-treatment relapses‡					
Mean ± SD	2.33±1.68	2.41±1.73	2.31±1.66	2.10±1.32	0.946
Median (Range)	2.0 (1–6)	2.0 (1–7)	2.0 (1–6)	2.0 (1–4)	
ARR	1.17	1.21	1.16	1.05	0.946
Patients -% with ≤1 relapse	40	45	40	35	
Baseline EDSS score‡					
Mean ± SD	2.52±1.23	2.15±1.05	2.42±1.21	2.39± 0.93	0.775
Median (Range)	2.5 (1.0–5.5)	2.0 (1.0–4.0)	2.5 (0.0–5.0)	2.5 (1.0–4.0)	
1b.					
Characteristics	Group A (n=10)	Group B† (n=10)	Group C (n=9)	Placebo (n=12)	P- value
Sex					
Female - no. (%)	5 (50)	7 (70)	6 (66.6)	10 (83.3)	0.419
Age (yr)					
Mean ± SD	36.6±13.5	34.80±5.4	40.9±8.1	39.8±13.2	0.572
Median (Range)	34.5 (22–65)	34.5 (26–43)	40.0 (29–54)	37.5 (21–58)	
Treatment history					
Patients on DMT- no. (%)	6 (60)	4 (40)	6 (67)	6 (50)	0.949
Pre-treatment disease duration (yr)					
Mean ± SD	9.7±10.0	8.3±5.3	11.3±6.1	8.7± 7.1	0.807
Median (Range)	7.5 (2–37)	8.0 (2–20)	8.0 (4–24)	5.5 (2–25)	
Pre-treatment relapses					

Mean ± SD	2.20±1.47	2.70±1.25	1.78±0.66	1.67±1.37	0.241
Median (Range)	2.0 (1 – 6)	2.5 (1 – 4)	2.0 (1 – 3)	1.5 (1 – 4)	
ARR	1.10	1.35	0.89	0.83	
Patients -% with ≤1 relapse	30	20	33	50	
Baseline EDSS score					
Mean ± SD	2.65±1.37	2.40±1.12	2.11±1.02	2.16±0.96	0.698
Median (Range)	3.0 (1.0–5.5)	2.5 (1.0–4.0)	2.0 (1.0–4.0)	2.0 (1.0–3.5)	

† PLP10 group

‡ Available data at Entry Baseline (n=18 for group A, n=17 for group B, n=19 for group C, n=19 for group D)

Table 1. The table section 1a reports the demographics and baseline disease characteristics for total randomized population by treatment arm.

The table section 1b reports the demographics and baseline disease characteristics of all-time on-study population by treatment arm. There were no significant between study-group differences at baseline for any characteristic.

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2a.								
Characteristics	Group A (N=10)		Group B† (N=10)		Group C (N=9)		Placebo (N=12)	
End Point	X	Y	X	Y	X	Y	X	Y
Total No. of relapses	22	17	27	8	16	13	20	25
Annual Relapse Rate (ARR)	1.10	0.85	1.35	0.40	0.88	0.72	0.83	1.04
% Reduction Compared to Placebo (primary endpoint) (Ys)¶	-18		-62		-30		N/A	
P Value against placebo	0.468		0.024		0.578			
ARR change -% (Y to X)¶	-23		-70		-18		+25	
P value against baseline	0.425		0.003		0.578		0.500	
X: Total number of relapses of 24 months pre-treatment (baseline) Y: Total number of relapses of 24 months on-treatment ¶ Unadjusted estimate								
2b.								
Excluding patients on natalizumab	Group A (N=9)		Group B† (N=10)		Group C (N=9)		Placebo (N=10)	
End Point	X	Y	X	Y	X	Y	X	Y
Total No. of relapses	16	15	27	8	16	13	13	19
Annual Relapse Rate (ARR)	0.88	0.83	1.35	0.40	0.88	0.72	0.65	0.95
% Reduction Compared to Placebo (primary endpoint) (Ys)¶	-13		-58		-24		N/A	
P Value against placebo	0.493		0.016		0.412			
ARR change -% (Y to X)¶	-6		-70		-18		+46	
P value against baseline	0.857		0.003		0.578		0.354	
X: Total number of relapses of 24 months pre-treatment (baseline) Y: Total number of relapses of 24 months on-treatment † PLP10 group ¶ Unadjusted estimate								
Table 2. The table section 2a reports the two year primary end points of ARR of all-time on-study population by treatment arm and percent difference with placebo. During the 24mo period on-treatment								

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3 the ARR of the group A was 0.85 with 18% decrease compared to placebo (p=0.468), of PLP10 group
4 0.40 with 62% decrease (p=0.024), and of group C 0.72 with 30% decrease (p=0.578) and reports the
5 comparison of the 24mo pre-treatment ARR (baseline ARR) to 24mo on-treatment ARR of all-time on-
6 study population including patients on natalizumab.
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14 The table section 2b reports comparison of the 24mo pre-treatment ARR to 24mo on-treatment ARR of
15 all-time on-study population excluding patients on natalizumab; and the comparison of the ARR during
16 the 24mo period on-treatment (primary end point) between each one of the groups against placebo.
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3a.								
Characteristics	Group A (N=8)		Group B† (N=7)		Group C (N=10)		Placebo (N=7)	
	X	Y	X	Y	X	Y	X	Y
No. of Relapses	20	14	14	14	27	26	20	13
Annual Relapse Rate (ARR)	1.25	0.88	1.00	1.00	1.35	1.30	1.42	0.92
X: Total number of relapses of 24 months pre-treatment Y: Total number of relapses of 24 months on-treatment								
3b.								
Characteristics	Group A (N=20)		Group B† (N=20)		Group C (N=20)		Placebo (N=20)	
End Point	X	Y	X	Y	X	Y	X	Y
No. of Relapses	45	34	49	30	46	41	43	41
Annual Relapse Rate (ARR)	1.13	0.85	1.23	0.75	1.15	1.03	1.08	1.03
ARR Reduction -% (Y to X)¶	-25		-39		-10		-5	
P value against baseline	0.120		0.005		0.475		0.652	
% Reduction of the ARR Compared to Placebo (Ys)¶	-18		-27		0.0		N/A	
P Value against placebo	0.447		0.121		0.996			
X: Total number of relapses of 24 months pre-treatment (baseline) Y: Total number of relapses of 24 months on-treatment ¶ Unadjusted estimate † PLP10 group								

Table 3. The table section 3a reports the two year primary end point of relapses based on study design as reported by drop-out patients by Treatment Arm. The most drop-out patients that went on disease modified therapy (DMT) were from the group A and placebo with three and two patients respectively on natalizumab. These parameters justify the decreased number of relapses recorded within group A and placebo drop-outs; and this could affect the ITT analysis in favor of placebo when the total two-year recorded data are used. For PLP10 group, 14 relapses were reported at baseline and remained the same during the two-year study. For placebo, 20 relapses were reported at baseline and decreased to 13 during the two-

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3 year study. This is expected since, for PLP10 group 43% of the drop-outs were under DMT at
4 entry baseline and remained the same until the end of the study with no patient on
5 natalizumab; but 57% of the placebo group drop-outs that were under DMT at entry baseline
6 increased to 86% at the end of the study including two patients on natalizumab.
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14 The table section 3b reports comparison of 24 months pre-treatment ARR (baseline) to 24
15 months on-treatment ARR of total randomized population, by treatment arm. The ARR of
16 PLP10 group was 1.23 at baseline and 0.75 at the end of the study (39% reduction $p=0.005$),
17 and for placebo 1.08 at baseline and 1.03 at the end of the study (5% reduction $p=0.652$). No
18 statistical difference was calculated for the other two treatment arms. During the 24 months
19 on-treatment, PLP10 group presented a 27% reduction of ARR vs. placebo ($p=0.121$), with all
20 groups without statistically significant results.
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Characteristics*	Group A (n=10)	Group B PLP10 (n=10)	Group C (n=9)	Placebo (n=12)	P-value Group B vs Placebo
Annual relapse rate over 1 year**	0.80	0.40	0.78	0.83	
Total number of relapses**	8	4	7	10	
Primary end points					
Annual relapse rate over 2 years (95% CI)**	0.85	0.40 (0.15-0.87)	0.72	1.04	0.024
Total number of relapses**	17	8	13	25	
Excluding patients on natalizumab					
Annual relapse rate over 2 years (95% CI)	0.83	0.40 (0.10-0.79)	0.72	0.95	0.016
Total number of relapses	15	8	13	19	
Secondary end points					
Cumulative probability of sustained progression increase by 1 point on EDSS confirmed after 6 mo, over 2 years -% **	43	10 (1/10)	24	58 (7/12)	0.019
Excluding patients on natalizumab					
cumulative probability of sustained progression increase by 1 point on EDSS confirmed after 6 mo, over 2 years -%	33	10 (1/10)	24	70 (7/10)	0.006
Exploratory Results					
Patients proportion with ≤1 relapse over 2 years -% **	50 (5/10)	90 (9/10)	56 (5/9)	42 (5/12)	0.030
MRI					
Patients proportion with new or enlarging T2 lesions-% **	----	29 (2/7)	----	67 (4/6)	
Excluding patients on natalizumab					
Patients proportion with no new or enlarging T2 lesions-%	----	29 (2/7)	----	80 (4/5)	
DMT (interferons, glatiramer acetate) and natalizumab					
Patients proportion on DMT and natalizumab at the end of 2 years-% **	80 (8/10)†	60 (6/10)	67 (6/9)	75 (9/12) ‡	0.747
* CI denotes confidence interval.					
** Including patients on natalizumab					
† 1 out of 10 on natalizumab					
‡ 2 out of 12 on natalizumab					

Table 4. Clinical end points, according to study group for all-time on-study population.

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33
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34 717 Ethical approval: Cyprus National bioethics committee (reference EEBK/EII/2005/10).
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38 719 All authors have completed the Unified Competing Interest form at
39
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23 735 Data Sharing:

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26 736 No additional unpublished data from the study are available
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Article Summary

Article focus:

- The increasing prevalence of Multiple Sclerosis (MS), the limited efficacy and the side effects of the existing treatments urge the clinical need for the development of new, innovative, more effective, safe, and preventive treatment strategies.
- For the first time we propose three original nutraceutical treatment intervention-cocktails, formulated based on systems medicine through nutritional systems biology rational; with PLP10 representing the complete composition of the formulation and the other two treatments represent partial formulations.
- We have studied the proposed interventions on 80 relapsing remitting MS patients (four

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3 groups of 20 each) for a total of 42 months, in a randomized, double-blind, placebo-
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5 controlled, phase II proof-of-concept clinical trial.
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10 **Key messages:**

- 11 • The per-protocol data analyses indicated that PLP10 is associated with significant
12 efficacy vs placebo on both reducing the annualized relapse rate and disease progression
13 without adverse or significant side effects.
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- 16 • PLP10 has probably the ability to interfere with the total known repertoire of mechanisms
17 involved in MS pathogenesis as well as with the recovery mechanisms, neuroprotection
18 and remyelination.; it is potentially able to manipulate global perturbed networks of the
19 disease causing factors rather than focusing only on unique failing components.
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- 22 • This proof-of-concept clinical study might be indicative and the beginning of new
23 avenues in MS prevention and treatment and of new epoch of novel drug development
24 with dynamic therapeutic potential for chronic complex multifactorial diseases.
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36 **Strengths and limitations of this study:**

- 37 • The strength of this study is the systems medicine therapeutic approach, the length of the
38 study the inclusion of the six months normalization (chronotherapy) period, and the study
39 protocol following all indicated appropriate guidelines with definite inclusion/exclusion
40 criteria and primary/secondary end points.
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- 43 • The sample size and the high rate of drop-outs (palatability of the formula) are the
44 limitations associated with the present study.
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- 47 • A phase III multi-center clinical trial will establish the present intervention regime among
48 the best choices among neurodegenerative diseases prophylaxis and MS treatment drugs
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13 885 **Figure legends**

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17 886 **Figure 1.** Omega-6 and omega-3 PUFA, their respective metabolic derivatives and their
18 possible effects on inflammation.
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22 888 After consumption, the PUFAs are metabolized via several pathways (not shown) to active
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24 889 compounds that mediate inflammation and products that promote resolution of inflammation.
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28 890 Abbreviations: PL, phospholipid; IFN- γ , interferon γ ; IL-2, interleukin 2; NF κ B, nuclear
29 factor kappa B; PGE₂, prostaglandin E₂; PPAR γ , peroxisome proliferator-activated receptor
30 γ ; PUFAs, polyunsaturated fatty acids; TGF β , transforming growth factor β ; TNF, tumor
31 necrosis factor; COX, cyclooxygenase; hydroxyeicosatetraenoic acid; HPETE,
32 hydroperoxyeicosatetraenoic acid; LOX, lipoxygenase; LT, leukotriene; PG, prostaglandin;
33 TX, thromboxane; RXR- γ , retinoid X receptor-gamma; Nrf2, nuclear respiratory factor;
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35 893 MMP, metalloproteinase.
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42 896 **Figure 2.** Study Flowchart
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48 898 **Figure 3.** Panel A demonstrates the ARR of all-time on-study patients during the 24mo pre-
49 treatment (baseline ARR) and at different on-study intervals (6, 12, 18, 24 mo) per treatment
50 arm. **
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55 901 Panel B demonstrates the ARR of all-time on-study population between 0-6, 6-12, 6-18, and
56 6-24 mo period intervals, of PLP10 vs. placebo group. **
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3 903 Panel C demonstrates the ARR of all-time on-study population of PLP10 vs. placebo group at
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5 904 baseline, during 1st year, and during the 2-year on-treatment. **
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8 905 Panel D demonstrates the dispersion of relapses throughout the 2-year period of all-time on-
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10 906 study (excluding patients on natalizumab) of PLP10 (n=10) vs. placebo (n=10). Placebo
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12 907 shows an irregular dispersion of relapses in relation to PLP10 group with linear increasing
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14 908 trend while PLP10 shows a stabilized linear trend. By using the per-protocol model where
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16 909 patients on natalizumab were excluded, we could compare the number of relapses on a same
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18 910 number of patients.
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22 911 ** Including the patients on natalizumab.
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25 912 **Figure 4.** Panel 4A demonstrates the Kaplan–Meier plot of the time to sustained progression
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27 913 of disability among all-time on-study patients, excluding patients on natalizumab, receiving
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29 914 intervention A, PLP10 and C as compared with placebo. PLP10 reduced the risk of sustained
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31 915 progression of disability by 86% over two years (p=0.006). Intervention formula A reduced
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33 916 the risk of sustained progression of disability by 53% (p=0.266) and intervention formula C
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35 917 by 67% (p=0.061).
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39 918 Panel 4B demonstrates the Kaplan–Meier plot of the time to sustained progression of
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41 919 disability among ITT population receiving intervention A, PLP10 and C as compared with
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43 920 placebo. PLP10 reduced the risk of sustained progression of disability by 71% over two years
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45 921 (p=0.052, trend). Intervention formula A reduced the risk of sustained progression of
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47 922 disability by 22% (p=0.727) and intervention formula C by 40% (p=0.447).
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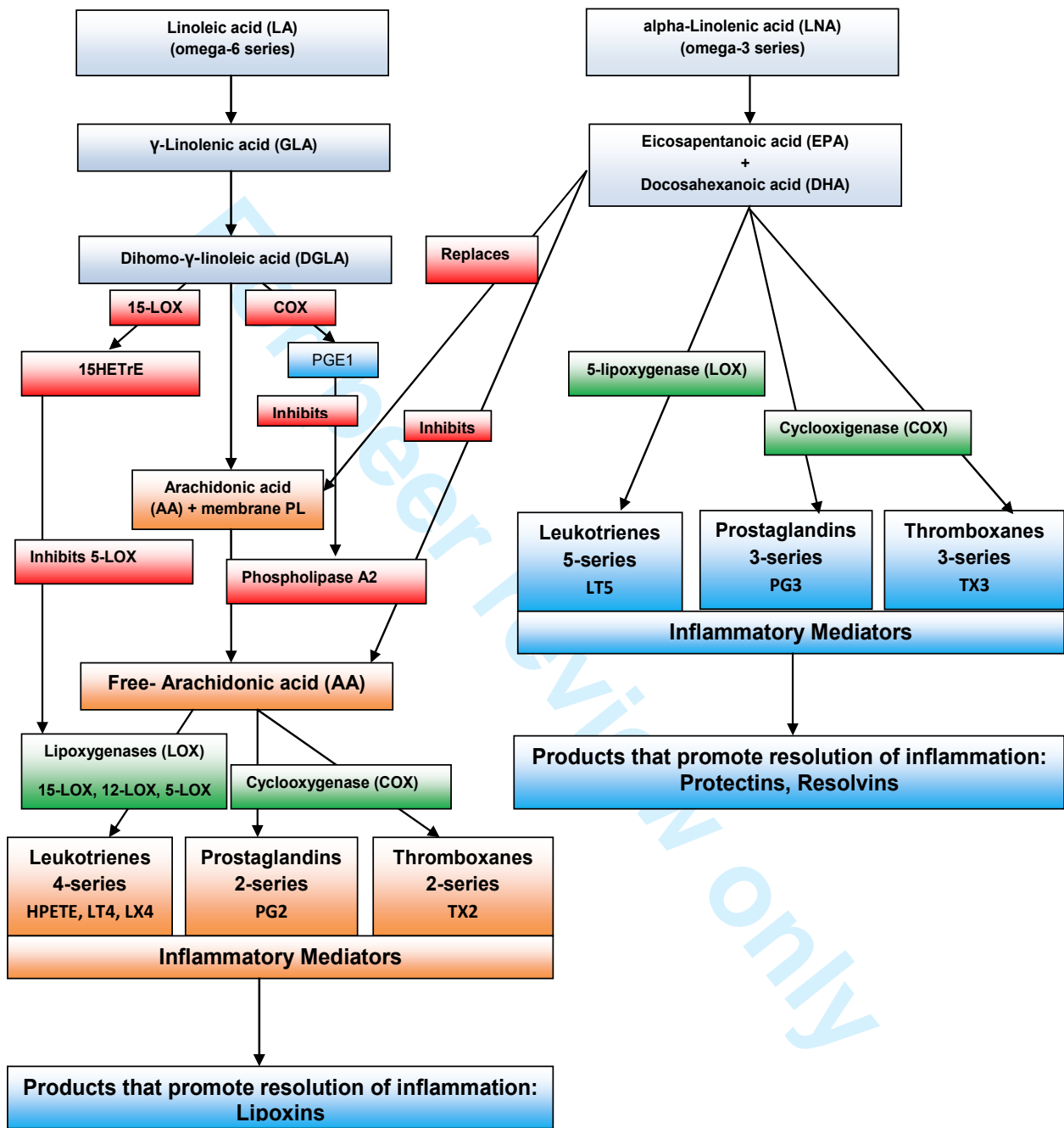
51 923 **Figure 5.** Mean change in expanded disability status scale score as a function of visit
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53 924 number. Values are expressed as mean ± s.e.m.
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56 925 ¶ Including patients on natalizumab
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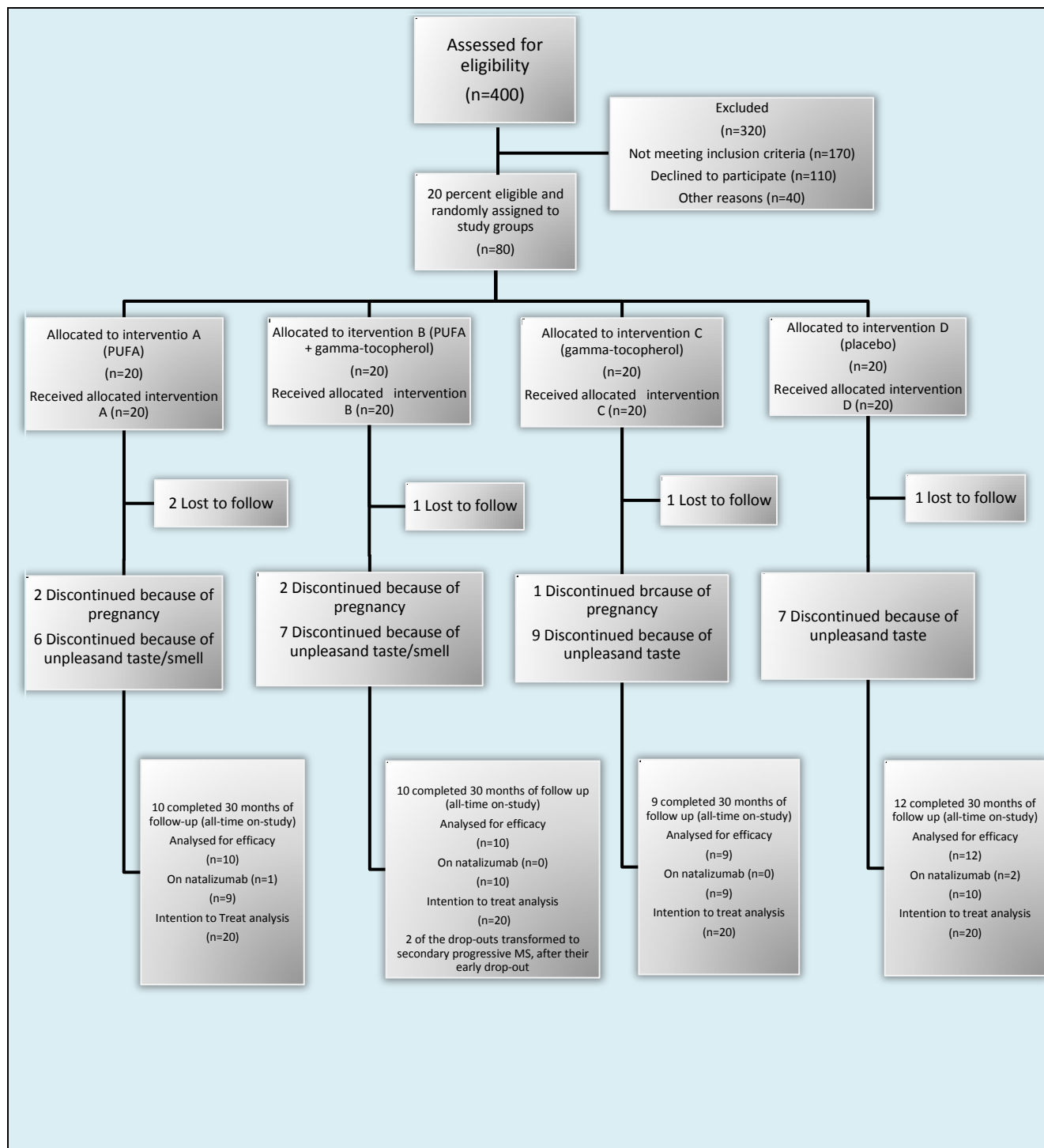
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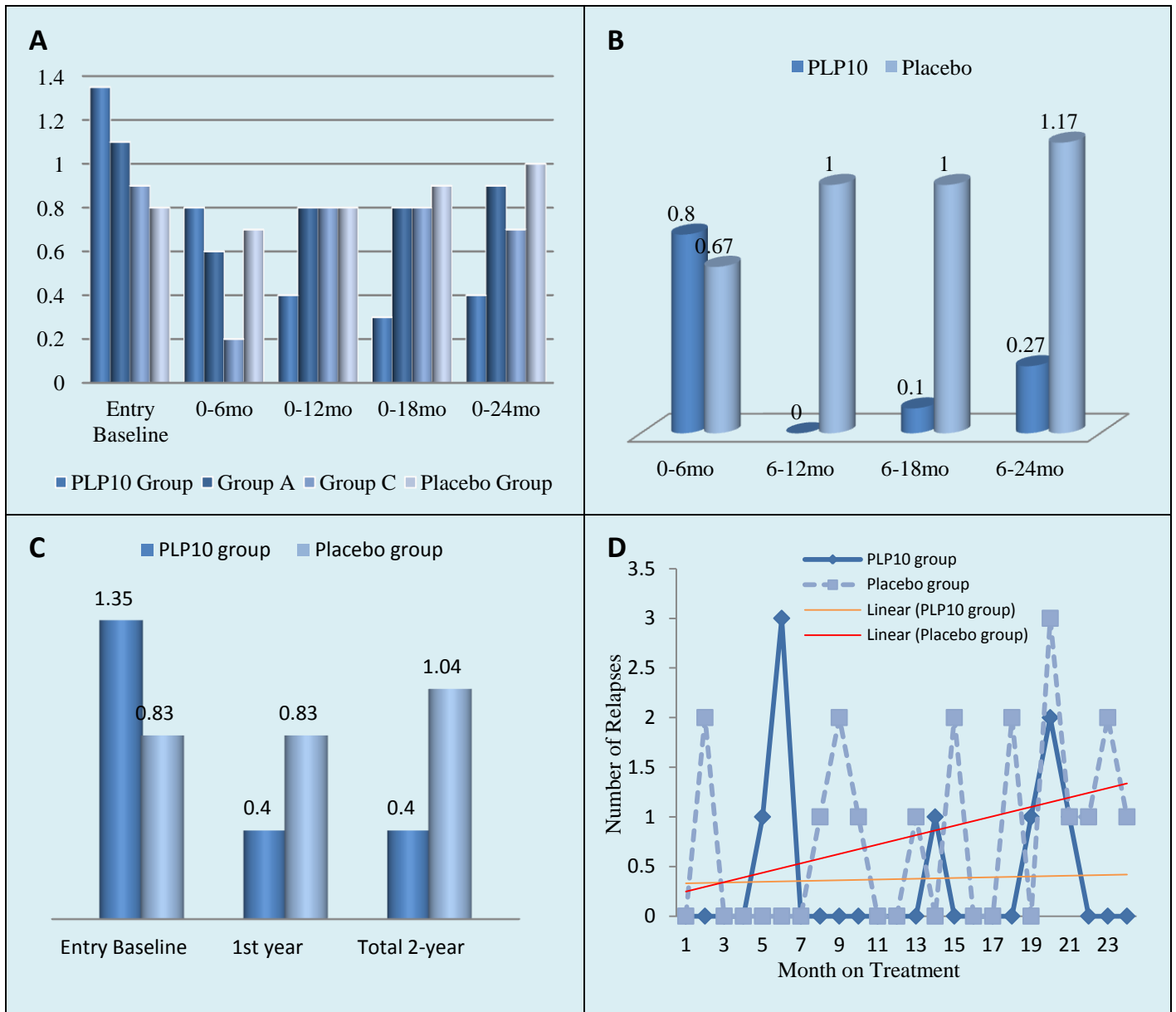
Omega-6 and omega-3 PUFA Consumption through Diet



Possible effects on inflammation:
 Reduce IFN- γ production; Reduce IL-2 production; Increase TGF β activity; Increase PGE2 activity; Inhibit arachidonic acid; Reduce TNF levels; RXR- γ and PPAR γ agonist; NF κ B expression; stimulate Nrf2; reduce MMP-2, -3, -9, and -13

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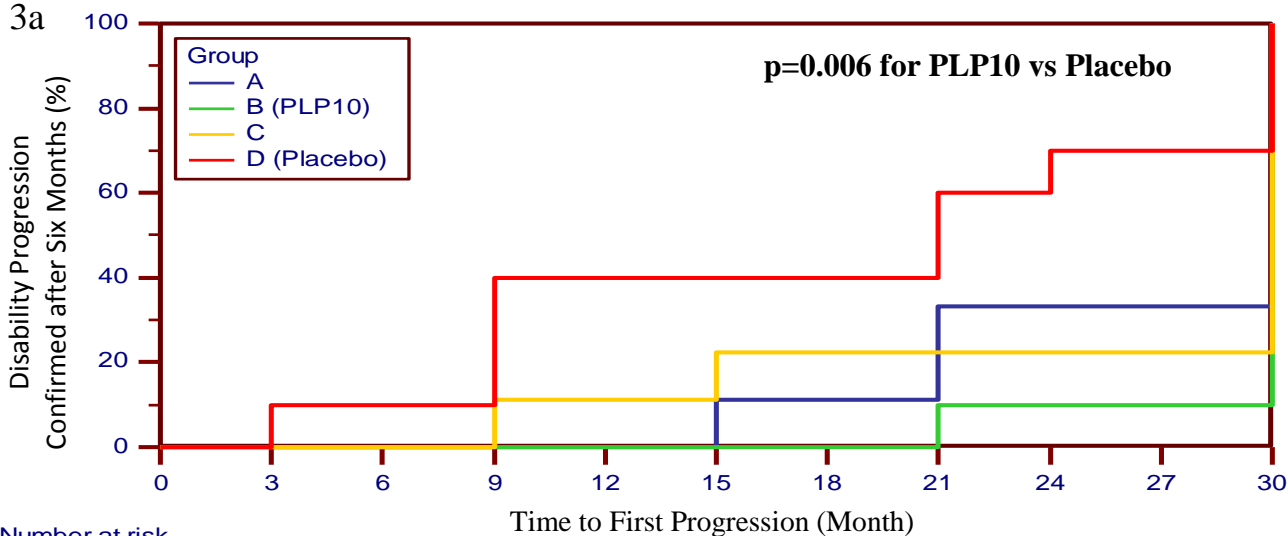




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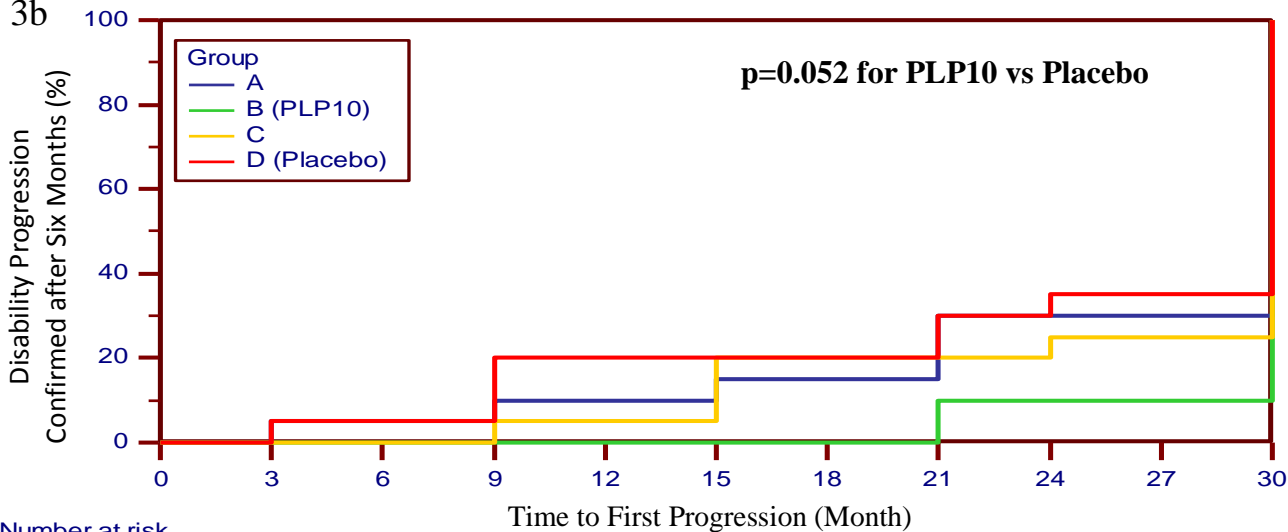
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Number at risk

Group: A	9	9	9	9	8	8	6	6	6	6
Group: B (PLP10)	10	10	10	10	10	10	9	9	9	9
Group: C	9	9	9	8	8	7	7	7	7	7
Group: D (Placebo)	10	9	9	6	6	6	4	3	3	3

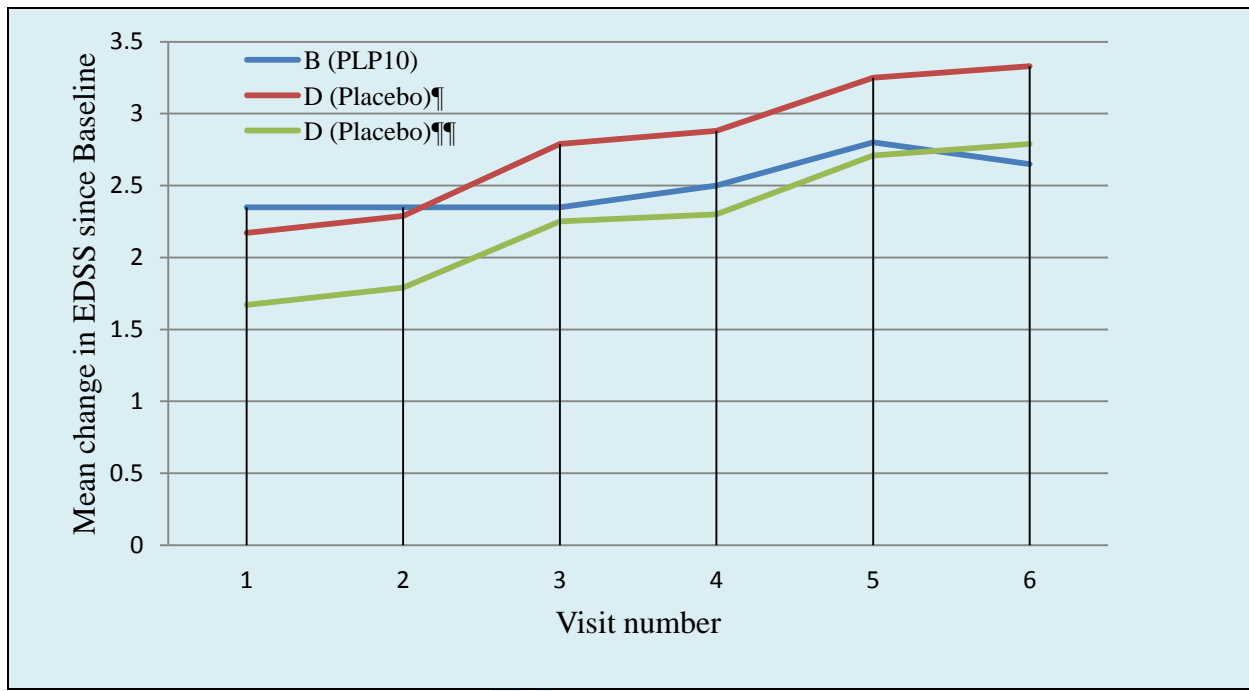
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Number at risk

Group: A	20	19	19	18	18	17	17	14	14	14	14
Group: B (PLP10)	20	20	20	20	20	20	20	18	18	18	18
Group: C	20	20	20	19	19	16	16	16	15	15	15
Group: D (Placebo)	20	19	19	16	16	16	16	14	13	13	13

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1 **Supplementary Information**

2 Table of Content

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Supplementary Information Methods 2	5-6
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34 **Supplementary Information Methods 1**

35 **Intervention and study rational.** We are using high dosage of 1:1 omega-3:omega-6
36 polyunsaturated (PUFA) aiming to overpass and normalize global diet regional traditions and
37 habits in relation to abnormal PUFA/saturated fatty acids (SFA)/monounsaturated fatty acids
38 (MUFA) daily ratio consumption irrespective of the quantities consumed; and enough to
39 equilibrate patients' diet in line with the recommended more physiologic omega-3:omega-6
40 ratio of about 1:1-4 wt/wt as reported by Simopoulos, 2002. In addition to correct existing
41 deficiencies, cell membrane abnormalities, specifically of the immunopathological system
42 and blood mononuclear peripheral cells, and high enough for availability and immediate
43 ongoing modulation of the involved pathogenic mechanisms and network of events in MS.
44 The high dosage is also required to overpass the quantity limitations, previously discussed, of
45 diet-consumed PUFAs for cellular incorporation, especially in the central nervous system
46 (CNS) of adults. Additionally, fatty acids (FAs) must first cross the intestinal epithelium
47 before reaching the different tissues, where digestion and absorption constitute further
48 problems in their availability (Carlier, H, 1991). Omega-3 PUFA are used in re-esterified
49 form to eliminate unwanted disturbances, at the sides of action, by other fatty acids and
50 molecules present in crude fish oils but also to increase the bioavailability of the FA since
51 triglycerides have been shown to be associated with much higher bioavailability (Dyerberg et
52 al, 2010). Linoleic (LA) and gamma linolenic acid (GLA) are essential structure molecules
53 and important for any physiological (re)generation of cell membrane. GLA quantity is
54 doubled to LA to ensure high direct production of dihomo-gamma-linolenic acid (DGLA),
55 from GLA when LA cannot be metabolized, due to desaturase deficiency or malfunction.
56 Such a reduced capacity to convert LA to GLA has been associated with aging, diabetes,
57 alcoholism, atopic dermatitis, premenstrual syndrome, rheumatoid arthritis, cancer and
58 cardiovascular diseases (Bolton-Smith et al, 1997; Horrobin, 1990; Leventhal et al, 1993).
59 This is going to result in the increase of DGLA relative to arachidonic acid (AA) with DGLA
60 promoting production of prostaglandin (PG)E1 but also inhibition of phospholipase (PL)A2:
61 two major reasons and rational for their use. If other metabolic problems are involved within
62 the omega-6 series and the normal metabolites are not produced then the eicosapentaenoic
63 acid EPA available in PLP10 will substitute the function of DGLA, as a competitive inhibitor
64 of AA for PLA2. In both cases the pro-inflammatory leucotrienes, prostaglandines of the 2-
65 series (PG2) and thromboxanes of the 2 series including the platelet-activator factor (PAF)
66 will be attenuated. The synthesis of AA from DGLA by $\Delta 5$ desaturase promoted by LA/GLA
67 supplementation is very limited in humans as a result of limited activity of the enzyme
68 (Yang-Yi & Robert, 1998). AA in the body is mostly available through diet. EPA and
69 docosahexaenoic acid (DHA) are both physiologically important and crucial structured
70 molecules able to substitute excess AA and SFA within the cell membranes. EPA will
71 contribute to the inhibition (competitive to AA) of PLA2, joining the co-supplied omega-6
72 PUFA but will also participate in the production of anti-inflammatory leukotrienes,
73 prostaglandins of the 3-series (PG3) and thromboxane (TX3) along with DHA, both found in
74 the PLP10 intervention. Moreover EPA will replace AA of the membrane phospholipids and
75 both omega-3 PUFA will contribute replacing abnormal quantities of SFA and excess AA.
76 DHA is used in 3:1 ratio to EPA to cover any possible inabilities of EPA to be metabolized,
77 high enough to strongly promote high production of the aforementioned anti-inflammatory

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3 78 eicosanoids and cytokines and to be incorporated into CNS cell membranes where DHA
4 79 should be the major PUFA present, replacing other FA, probably saturated and excess of AA.
5 80 EPA, DHA, LA and GLA along with the rest of the other ingredients used (“other” omega-3
6 81 PUFA, SFA and MUFA that are usually found in the cell membranes of healthy people in
7 82 limited quantities) in the intervention regimen are for their availability as minor structural
8 83 constituents of physiological cellular membranes integrity, fluidity and overall function as
9 84 building blocks for myelin repair and/or myelination. Furthermore the PUFA used within the
10 85 cocktail intervention aimed to manipulate all other pathophysiological pathways that are
11 86 reported to be able to: as previously discussed including gene transcription for
12 87 neuroprotection and remyelination. Furthermore, PUFA are used to ensure the integrity of
13 88 blood brain barrier (BBB) and to modulate the gelatinases responsible for the T cell migration
14 89 within the CNS. Three different antioxidant vitamins (vitamin E, mostly as alpha-tocopherol,
15 90 gamma (γ)-tocopherol (vitamin E isoform) and vitamin A) are used in the regimen
16 91 preparation to support the cellular antioxidant defenses but also to protect peroxidation of the
17 92 supplied increased amounts of PUFA. Alpha-tocopherol low-molecular-weight antioxidants
18 93 will contribute to radical scavenging, interfering with gene transcription, protein expression,
19 94 enzyme activity and metal chelation (van Meeteren et al, 2005). Vitamin E (alpha tocopherol)
20 95 and vitamin A are used as antioxidants for the protection of the excess supplemented PUFA,
21 96 with alpha-tocopherol been demonstrated to protect against peroxynitrite-induced oxidative
22 97 damage, as well as able to efficiently detoxify hydroxyl, perhydroxyl and superoxide free
23 98 radicals (the elevated reactive oxygen species (ROS)); each one with different mechanism of
24 99 action, increasing the effect capability (Vatassery et al, 1998b; van Meeteren, 2005). Gamma-
25 100 tocopherol is used in high dosage since its half life is very short compared to alpha-
26 101 tocopherol and has been demonstrated to specifically protect against nitro-radicals.
27 102 Tocopherols can also exert non-antioxidant properties, including modulation of cell signaling
28 103 and immune function, regulation of transcription, and induction of apoptosis as previously
29 104 discussed (van Meeteren et al, 2005).

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39 105 PLP10 is the first preparation ever developed for MS therapy that is composed by the use of
40 106 all different previously discussed PUFA, MUFA, SFA in a cocktail preparation mixed with
41 107 the specific aforementioned antioxidant vitamins that have never been all together used
42 108 before within a specific formulation. The ingredients ratio, quality, structural form and
43 109 mostly the high dosage has never been before tested. Furthermore, the knowledge and
44 110 chronotherapy as well as other unique limitations associated with the individual molecules
45 111 used, have never been accounted, discussed, proposed or reported for any previous
46 112 therapeutic regimen.

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50 113 Through systems medicine therapeutic philosophy, by the use of PLP10, potentially MS
51 114 patients have the opportunity to be treated holistically, by natural source isolated molecules,
52 115 demonstrated as able of affecting and modulating all known pathophysiological,
53 116 immunopathological, habitual, gene related factors; thus the dynamic interconnected complex
54 117 network of events simultaneously. Possibly synergistic effects between PLP10 ingredients are
55 118 also feasible. Moreover we can speculate that treatment efficacy of PLP10, when used as

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3 119 adjunct to existing pharmaceuticals produced by reductionism, can be proven therapeutically
4 120 superior to any available treatment for MS.
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160 **Supplementary Information Methods 2**

161 **Interventions specifications.** The specific omega-3 (re-esterified glycerides) and omega-6
162 (glycerides) raw materials were purchased according to the required interventions' PUFA-
163 fraction specification (molecular structure, quantity/ratio and quality) with vitamin E (alpha-
164 tocopherol) used as antioxidant stabilizer by the supplier. The vitamins and masking aroma
165 were purchased separately. The mixing of fractions to the final required intervention-
166 composition specification was always performed by the same team of scientists under the
167 supervision of the involved medical biochemist and lipidology specialist, under appropriate
168 conditions every six months. Interventions were stored refrigerated in dark until use.

169
170 The ratios of the different ingredients used were as follows: omega-3, EPA to DHA (about 1
171 to 3 wt/wt), omega-6, LA to GLA (about 2 to 1 wt/wt), omega-3 (EPA + DHA) to omega-6
172 (LA + GLA) (about 1 to 1 wt/wt). The total omega-3 (EPA + DHA + "other" omega-3
173 PUFA) used as re-esterified triglycerol (minimum value 60%), diglyceride (about 33%),
174 monoglyceride (about 2%) structural form mixture and about 2% ethyl ester structural form,
175 with no less than 80% re-esterified triglycerol content to be DHA and EPA as a result of
176 PUFA triglycerides re-esterification of fish body oils. The "other" group of omega-3 on the
177 re-esterified glycerols included the 18:3 (alpha-linolenic acid), 18:4 (stearidonic acid), 20:4
178 (eicosatetraenoic acid) and 22:5 (docosapentaenoic acid) PUFA. The fraction of omega-6
179 (LA + GLA) used as triglycerides with no less than 50-65% triglycerol content to be LA and
180 GLA in a ratio of 2 to 1 with 18:1 (oleic acid) 14-20% and 20:1 (eicosenoic acid), 22:1
181 (docosenoic acid), 24:1 (tetracosenic acid) as additional monounsaturated fatty acids and
182 minor quantities of 16:0 (palmitic acid) 4-16%, 18:0 (stearic acid) 2-5% saturated fatty acids
183 from Borage oil source. The vitamins used were vitamin A as beta-carotene, vitamin E
184 (alpha-tocopherol) and pure gamma-tocopherol (vitamine E isoform). Citrus extract was used
185 as masking aroma and pure virgin olive oil as delivery vehicle.

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187 **The daily intervention formula agent dosages were:**

188 **Intervention formula A** daily dosage: EPA (1650mg) / DHA (4650mg) / GLA (2000mg) /
189 LA (3850mg) / total other omega-3 (600mg) / total monounsaturated fatty acids (MUFA)
190 (18:1 1300mg, 20:1 250mg, 22:1 82mg, 24:1 82mg) + total saturated fatty acids (SFA) (18:0
191 160mg, 16:0 650mg) / vitamin A (0.6mg) / vitamin E (22mg).

192 **Intervention formula B (PLP10)** daily dosage: EPA (1650mg) / DHA (4650mg) / GLA
193 (2000mg) / LA (3850mg) / total other omega-3 (600mg) / total MUFA (18:1 1300mg, 20:1
194 250mg, 22:1 82mg, 24:1 82mg) + total SFA (18:0 160mg, 16:0 650mg) / vitamin A (0.6mg) /
195 vitamin E (22mg) / gamma- tocopherol (γ -tocopherol) (760 mg).

196 **Intervention formula C** daily dosage: γ -tocopherol (760 mg) (in 16137 mg pure virgin olive
197 oil as a vehicle).

198 **Intervention formula D** daily dosage: pure virgin olive oil (16930mg).

199 Citrus aroma was added in each intervention formula to make up a total dosage of 19.5ml of
200 solution per day.

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3 201 The specific omega-3 related fraction, according to specifications required for the
4 202 interventions was prepared and purchased from EPAX AS, Aalesund, Norway; as re-
5 203 esterified glycerides from fish body oils as a source. The specific omega-6 PUFA, MUFA
6 204 and SFA related fraction, according to required specifications, was prepared and purchased
7 205 from Goerlich Pharma International GmbH, Edling, Germany, as triglycerides from Borage
8 206 seed oil (organic, cold pressed) "*Borago officinalis*" as a source. Both omega-3 and omega-6
9 207 fractions were delivered stabilized by the producer (vitamine E (alpha-tocopherol) ~ 4.5 mg/g
10 208 was used as antioxidant).

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14 209 Vitamins: vitamin A as beta-carotene (HealthAid Ltd., Middlesex, United Kingdom) and pure
15 210 gamma-tocopherol (Tama Biochemical Co. Ltd., Shinjuku-ku Tokyo, Japan).

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18 211 Citrus aroma (Givaudan Schwaiz AG, Dubendorf, Switzerland).

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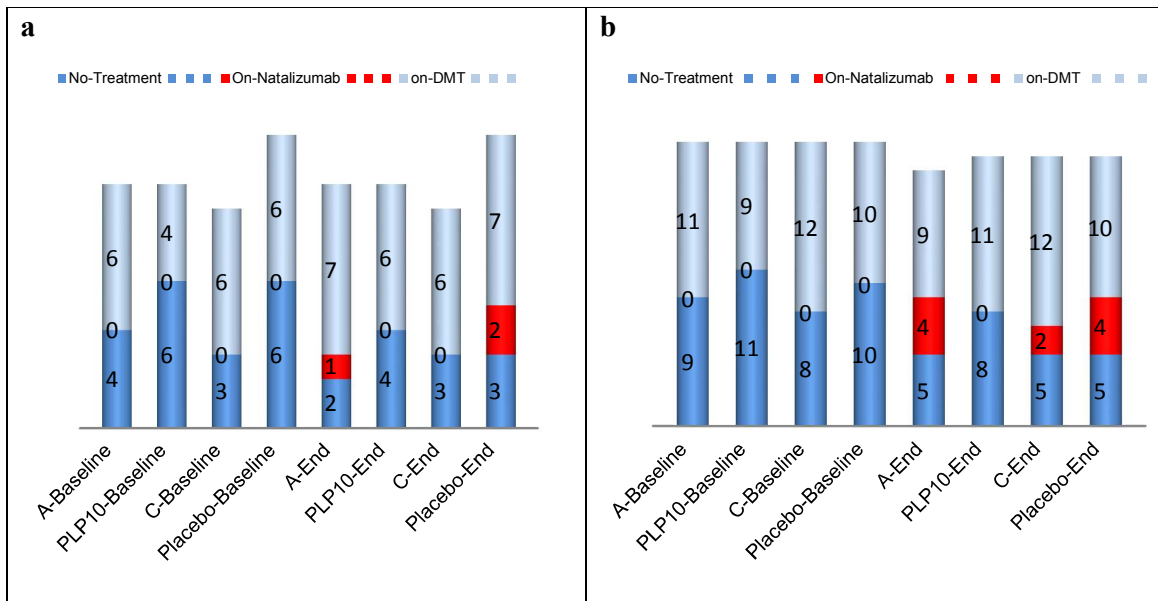
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Supplementary Information Figure 1 | Population on DMT and/or natalizumab. (a) Demonstrates the all-time on-study population per treatment arm that was receiving or not receiving DMT at entry baseline and the same population at the end of the trial (including patients on natalizumab). No statistical significant differences were calculated. The all-time on-study patients per treatment-arm that were receiving DMT at entry baseline were six patients out of ten (60%) within group A, four out of ten (40%) within PLP10 group, six out of nine (66%) within group C and six out of 12 (50%) within placebo. When the study completed, 80% of the patients in group A, 60% in PLP10 group, 66% in group C and 75% of the patients in placebo ended up on treatment. Within group A one out of eight and within placebo two out of nine patients on DMT transferred on natalizumab. (b) Demonstrates the total randomized population per treatment arm that was receiving or not receiving DMT at entry baseline and the same population at the end of the trial without lost to follow-up (including patients on natalizumab). A total of 61% of group A patients were on DMT at entry baseline and became 72% at the end; for PLP10 group 41% and became 53%; for group C 73% and became 74% and for placebo 53% and became 74% at study completion. At the end: for group A, four out of 13 patients on DMT transferred on natalizumab; for PLP10 group no patient was on natalizumab; for Group C two out of 14 patients on DMT transferred on natalizumab; and for placebo group four out of the 14 patients on DMT transferred on natalizumab. No significant differences measured at entry baseline between the groups.

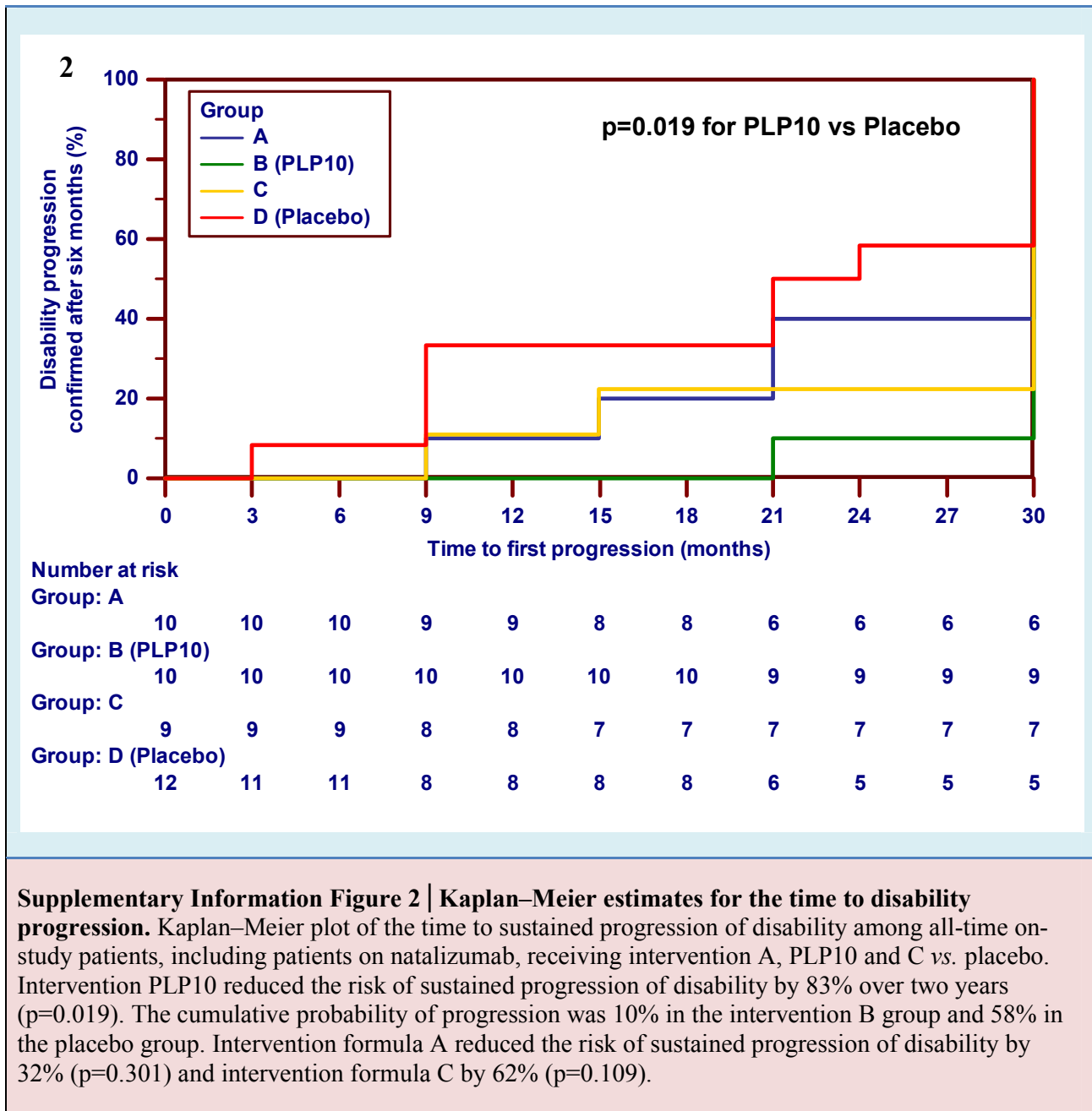
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Checklist of Items for Reporting Trials of Nonpharmacologic Treatments*

Section	Item	Standard CONSORT Description	Extension for Nonpharmacologic Trials	Reported on Page No.
Title and abstract†	1	How participants were allocated to interventions (e.g., “random allocation,” “randomized,” or “randomly assigned”)	In the abstract, description of the experimental treatment, comparator, care providers, centers, and blinding status	1,3,4
Introduction				
Background	2	Scientific background and explanation of rationale		5 to 8
Methods				
Participants†	3	Eligibility criteria for participants and the settings and locations where the data were collected	When applicable, eligibility criteria for centers and those performing the interventions	8, 13,15
Interventions†	4	Precise details of the interventions intended for each group and how and when they were actually administered	Precise details of both the experimental treatment and comparator	9,10,11 Appendix p. 5,6
	4A		Description of the different components of the interventions and, when applicable, descriptions of the procedure for tailoring the interventions to individual participants	10, Appendix p.5
	4B		Details of how the interventions were standardized	9,10,Appendix p.5
	4C		Details of how adherence of care providers with the protocol was assessed or enhanced	9
Objectives	5	Specific objectives and hypotheses		7,8
Outcomes	6	Clearly defined primary and secondary outcome measures and, when applicable, any methods used to enhance the quality of measurements (e.g., multiple observations, training of assessors)		12
Sample size†	7	How sample size was determined and, when applicable, explanation of any interim analyses and stopping rules	When applicable, details of whether and how the clustering by care providers or centers was addressed	13

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5	Randomization– sequence generation†	8	Method used to generate the random allocation sequence, including details of any restriction (e.g., blocking, stratification)	When applicable, how care providers were allocated to each trial group
6				9
7	Allocation concealment	9	Method used to implement the random allocation sequence (e.g., numbered containers or central telephone), clarifying whether the sequence was concealed until interventions were assigned	9
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11	Implementation	10	Who generated the allocation sequence, who enrolled participants, and who assigned participants to their groups	9
12				
13	Blinding (masking)†	11A	Whether or not participants, those administering the interventions, and those assessing the outcomes were blinded to group assignment	Whether or not those administering co- interventions were blinded to group assignment
14				9,10
15				
16		11B		If blinded, method of blinding and description of the similarity of interventions†
17				9,10,Appendix p.5
18	Statistical methods†	12	Statistical methods used to compare groups for primary outcome(s); methods for additional analyses, such as subgroup analyses and adjusted analyses	When applicable, details of whether and how the clustering by care providers or centers was addressed
19				13
20				
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23				
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26	Results			
27	Participant flow†	13	Flow of participants through each stage (a diagram is strongly recommended)--- specifically, for each group, report the numbers of participants randomly assigned, receiving intended treatment, completing the study protocol, and analyzed for the primary outcome; describe deviations from study as planned, together with reasons	The number of care providers or centers performing the intervention in each group and the number of patients treated by each care provider or in each center
28				15 Fig 2
29				
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35	Implementation of intervention†	New item		Details of the experimental treatment and comparator as they were implemented
36				10,15,16 Appendix p..5,
37	Recruitment	14	Dates defining the periods of recruitment and follow-up	15,11
38				
39	Baseline data†	15	Baseline demographic and clinical characteristics of each group	When applicable, a description of care providers (case volume, qualification, expertise, etc.) and centers (volume) in each group
40				15,Table 1
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Numbers analyzed	16	Number of participants (denominator) in each group included in each analysis and whether analysis was by “intention-to-treat”; state the results in absolute numbers when feasible (e.g., 10/20, not 50%)		14,15,16
Outcomes and estimation	17	For each primary and secondary outcome, a summary of results for each group and the estimated effect size and its precision (e.g., 95% confidence interval)		15 to 20
Ancillary analyses	18	Address multiplicity by reporting any other analyses performed, including subgroup analyses and adjusted analyses, indicating those prespecified and those exploratory		15 to 20
Adverse events	19	All important adverse events or side effects in each intervention group		20
Discussion				
Interpretation†	20	Interpretation of the results, taking into account study hypotheses, sources of potential bias or imprecision, and the dangers associated with multiplicity of analyses and outcomes	In addition, take into account the choice of the comparator, lack of or partial blinding, and unequal expertise of care providers or centers in each group	20,21
Generalizability†	21	Generalizability (external validity) of the trial findings	Generalizability (external validity) of the trial findings according to the intervention, comparators, patients, and care providers and centers involved in the trial	22
Overall evidence	22	General interpretation of the results in the context of current evidence		22 to 26

*Additions or modifications to the CONSORT checklist. CONSORT = Consolidated Standards of Reporting Trials.

†This item was modified in the 2007 revised version of the CONSORT checklist.



A novel oral nutraceutical formula (PLP10) for the treatment of relapsing remitting multiple sclerosis: a randomized, double-blind, placebo-controlled proof-of-concept clinical trial

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1 **A novel oral nutraceutical formula (PLP10) for the**
2 **treatment of relapsing remitting multiple sclerosis: a**
3 **randomized, double-blind, placebo-controlled proof-of-**
4 **concept clinical trial**

5 **Marios C. Pantzaris, George N. Loukaides, Evangelia E. Ntzani, Ioannis S.**
6 **Patrikios**

7 The Cyprus Institute of Neurology and Genetics (CING) 1683 Nicosia, Cyprus Marios C.
8 Pantzaris Senior Consultant Neurologist Neurology Clinic C and PALUPA Medical Ltd., The
9 Cyprus Institute of Neurology and Genetics (CING) 1683 Nicosia, Cyprus George N.
10 Loukaides Clinical Dietitian/Nutritionist Neurology Clinic and PALUPA Medical Ltd.,
11 University of Ioannina School of Medicine (UISM) 45110 Ioannina, Greece Evangelia E.
12 Ntzani Assistant Professor Clinical and Molecular Epidemiology Unit, Department of
13 Hygiene and Epidemiology, PALUPA Medical Ltd at The Cyprus Institute of Neurology and
14 Genetics (CING) 1683 Nicosia, Cyprus Ioannis S. Patrikios Senior Research Scientist,
15 visiting Professor at European University Cyprus Nicosia, Cyprus, Department of Health and
16 Science. Correspondence should be addresses to e-mail: I.Patrikios@euc.ac.cy or
17 pantzari@cing.ac.cy

18
19 **Correspondence to:**

20 **Ioannis Patrikios**

21 The Cyprus Institute of Neurology and Genetics (CING)
22 Neurology Clinic C (PALUPA Medical),

1
2
3 23 6 International Airport Av.
4
5 24 P.O.Box 23462, 1683 Ayios Dometios. Nicosia, Cyprus
6
7 25 Tel: +357 22 358 600, +357 99 097 856;
8
9
10 26 i.patrikios@euc.ac.cy
11
12 27 patrikiosioannis@gmail.com
13
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29 **AND**
30

31 **Marios Pantzaris**

32 The Cyprus Institute of Neurology and Genetics (CING)
33 Neurology Clinic C (PALUPA Medical),
34 6 International Airport Av.
35 P.O.Box 23462, 1683 Ayios Dometios, Nicosia Cyprus
36 Tel: +357 22 358 600;
37 pantzari@cing.ac.cy
38

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40 medicine, randomized clinical trial.
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46 **Word Count: 6415**
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3 **Abstract**
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5 **Objective** To assess whether our three novel interventions, formulated based on systems
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medicine therapeutic concept reduce disease activity in patients with relapsing remitting multiple sclerosis who were either treated with disease modifying treatment or untreated.

Design 30-month randomized double-blind, placebo-controlled, parallel design, phase II proof-of-concept clinical study.

Settings Cyprus Institute of Neurology and Genetics (CING)

Participants and Interventions 80 subjects were randomized into four groups of 20. The first intervention (A) was composed of omega-3 and omega-6 polyunsaturated fatty acids at 1:1 wt/wt. Specifically, the omega-3 fatty acids were docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) at 3:1 wt/wt and the omega-6 fatty acids were linoleic acid (LA) and gamma (γ)-linolenic acid (GLA) at 2:1 wt/wt. This intervention also included minor quantities of other specific polyunsaturated, monounsaturated and specific saturated fatty acids as well as vitamin A and vitamin E (alpha-tocopherol). The third intervention (C) was γ -tocopherol alone. The second intervention (PLP10) was a combination of A and C. A fourth group of 20 received a vehicle placebo. The interventions were administered per os once daily.

Main outcome measures The primary endpoint was the annualized relapse rate (ARR) of the three interventions versus placebo at two years. The secondary end point was the time to confirmed disability progression at two years.

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3 72 **Results** The per-protocol, proof-of-concept, analysis demonstrated a 64% adjusted relative
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5 73 reduction in ARR at two years for PLP10 versus placebo (P=0.024). Regarding the secondary
6
7 74 endpoint, a relative reduction of 86% in the risk of sustained progression of disability was
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10 75 observed within the PLP10 group (p=0.047). No adverse events were reported. Interventions
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12 76 A and C showed no significant efficacy.
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16 78 **Conclusions** PLP10 treatment significantly reduced the ARR, and the risk of sustained
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18 79 disability progression without any adverse or severe side effects.
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23 81 **Trial registration** International Standard Randomized Controlled Trial, number
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25 82 ISRCTN87818535.
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92 Introduction

93 Multiple sclerosis (MS) is a complex multifactorial disease that results from interplay
94 between as yet unidentified environmental factors and susceptibility genes.¹⁻³ Together, these
95 factors trigger a cascade of events, involving engagement of the immune system,
96 inflammatory injury of myelin, axons and glia, functional recovery and structural repair,
97 gliosis, and neurodegeneration.⁴ The bio-mechanisms involved are: immune-mediated
98 inflammation, oxidative stress and excitotoxicity.⁵⁻⁹ These mechanisms may all contribute to
99 oligodendrocyte and neuronal damage and even cell death, hence promoting disease
100 progression. The increasing prevalence of MS, the limited efficacy and the side effects of the
101 existing treatments urge the clinical need for the development of new, innovative, more
102 effective, safe, and preventive treatment strategies.

103
104 Research has shown that multiple variables dynamically interact and many different complex
105 interrelated processes are simultaneously orchestrated for MS pathogenesis. The fundamental
106 distinctiveness of systems medicine (SM) is not just the recognition that different specific
107 complex factors are important in disease management, but that they need to be incorporated
108 in some meaningful way to treatment selection and delivery.¹⁰ The primary challenge tackled
109 by systems scientific approach is the elucidation of how these multiple variables dynamically
110 interact and how one can apply this understanding to affect the system and achieve a
111 desirable end.¹⁰ The answer might be the simultaneous interference with all involved
112 perturbed mechanisms, by using a cocktail of different specific ingredients, potentially able
113 through synergistic effect to give a long, holistic and effective treatment (Supplementary
114 Information Methods 1).

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3 116 The polyunsaturated fatty acids (PUFA) composition of membrane phospholipids plays a
4
5 117 direct role in immune and non-immune related inflammation. PUFA and antioxidant
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7 118 deficiencies along with decreased cellular antioxidant defense mechanisms have been
8
9 119 reported for MS patients.¹¹The cause of these PUFA deficiencies is not entirely clear and may
10
11 120 involve metabolic and nutritional alterations.¹¹
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16 122 Increased or uncontrolled inflammation contributes to several different acute and chronic
17
18 123 diseases and it is characterized by the production of inflammatory cytokines, arachidonic acid
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20 124 (AA)-derived eicosanoids (prostaglandins (PGs), thromboxanes (TXs), leukotrienes (LTs),
21
22 125 and other oxidized derivatives), other inflammatory agents such as reactive oxygen species
23
24 126 (ROS) , nitric oxide (NO), and adhesion molecules (Fig 1).¹² During inflammation glutamate
25
26 127 homeostasis is altered by activated immune cells releasing increased quantities of glutamate
27
28 128 that can result in over activation of glutamate receptors and in return excitotoxic
29
30 129 oligodendroglial death.^{7, 13} As such, among others, membrane-related pathology, immune-
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32 130 mediated inflammation, oxidative stress and excitotoxicity provide potentially useful
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34 131 combined targets for intervention in MS.
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40 133 *In vitro and in vivo* studies have demonstrated that dietary EPA, DHA, LA, and GLA can be
41
42 134 implicated and modulate almost all known complex network of events and pathways
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44 135 repertoire in MS pathophysiology. Brain membrane fatty acid composition can be modified
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46 136 with dietary supplementation, but the process has been showed to be age dependent (it takes
47
48 137 much longer in adults versus developing brains) as well as possibly dependent on the
49
50 138 quantities of the dietary/supplemented PUFAs.¹⁴ Both human and animal studies proved that
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52 139 diets high in DHA and EPA increase the proportion of these PUFA in the membranes of
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54 140 inflammatory cells and reduce the levels of AA.^{12, 15} The anti-inflammatory properties of
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3 141 omega-3 include production of PGs and TXs of the 3-series and LTs of the 5-series (Fig 1).¹⁴
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5 142 ¹⁶ Resolvins and protectins are biosynthesized from omega-3 fatty acids via cyclooxygenase-
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7 143 2/lipoxygenase (COX-2/LOX) pathways and they promote control of inflammation in neural
8
9 144 tissues (Fig 1).¹⁷⁻²¹ T-cell proliferation in acute and chronic inflammation can be reduced by
10
11 145 supplementation with either omega-6 or omega-3 PUFA.²² Furthermore, vitamin E is an
12
13 146 important antioxidant that can interrupt the propagation of free radical chain reactions.²³
14
15 147 Vitamin E (alpha-tocopherol, an isoform of vitamin E) efficiently detoxifies hydroxyl,
16
17 148 perhydroxyl and superoxide free radicals.²⁴ However γ -tocopherol (another isoform of
18
19 149 vitamin E) seems to be more efficiently implicated in trapping NO radicals.²⁵ In addition
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21 150 alpha-tocopherol exerts non-antioxidant properties, including modulation of cell signaling
22
23 151 and immune function, regulation of transcription, and induction of apoptosis.²⁶
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27 153 Moreover, omega-3 fatty acids electrophilic derivatives formed by COX-2, in activated
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29 154 macrophages can stimulate the nuclear respiratory factor (Nrf2) inducing the transcription of
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31 155 neuroprotective and antioxidant related genes, and can activate the peroxisome proliferator-
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33 156 activated receptor (PPAR) γ for anti-inflammatory response.²⁷⁻²⁹ In animal studies, EPA and
34
35 157 DHA proved to be endogenous ligands of RXRs, with positive effect on neurogenesis.³⁰
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37 158 Additionally, in 2008 Salvati and coworkers reported evidense of accelerated myelination in
38
39 159 DHA- and EPA-treated animals.³² Moreover, DHA and EPA are reported to significantly
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41 160 decrease the levels of metalloproteinases (MMP) -2, -3, -9, and -13 with a significant role in
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43 161 the migration of lymphocytes into the CNS by inducing disruption of the blood brain barrier
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45 162 (BBB), an important step in the formation of MS lesions.³³⁻³⁹
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49 164 Based on the above, specific PUFA and antioxidant vitamins fulfill the criterion of biologic
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51 165 plausibility and have the potential to diminish MS symptoms severity and activity, even
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3 166 promoting recovery (remyelination).¹¹ Overall, PLP10 includes multiple ingredients
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5 167 interacting with key interconnected components within functional network modules, each
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7 168 contributing a fraction of the effects of perturbations that cause the disease.⁴⁰
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12 170 In our phase II, single-center, randomized, double-blind, placebo-controlled, proof-of-
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14 171 concept clinical trial we intended to evaluate the therapeutic ability of PLP10 and of two
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16 172 other interventions (A and C) consisting of PLP10 constituent partial fractions versus
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18 173 placebo, when used on RRMS patients.
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24 25 26 176 **Methods**

27 28 177 **Patients**

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30 178 Eligibility criteria were an age of 18 to 65; a diagnosis of RRMS according to the McDonald
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32 179 criteria; a score of 0.0 to 5.5 on the EDSS, a rating that ranges from 0 to 10, with higher
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34 180 scores indicating more severe disability; MRI showing lesions consistent with MS; and at
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36 181 least one documented clinical relapse either receiving or not disease modifying treatment
37
38 182 (DMT) within the 24 months period before beginning (enrollment) of the study. Patients were
39
40 183 excluded because of a recent (<30 days) relapse, prior immunosuppressant or monoclonal
41
42 184 antibodies therapy, pregnancy or nursing, other severe disease compromising organ function,
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44 185 progressive MS, history of recent drug or alcohol abuse, use of any additional food
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46 186 supplement, vitamin, or any form of PUFA, history of severe allergic or anaphylactic
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48 187 reactions or known specific nutritional hypersensitivity. No monitor or limitations on
49
50 188 patients' daily diet habits were included in the study design since the quantities of the
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52 189 ingredients within the formulas daily-dosage could not be significantly affected or spoiled by
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3 190 any confounding factors within any known global daily food diet (see procedures, treatment
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5 191 regimen and end-points).
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9 193 The study was conducted in accordance with the standards of the International Conference of
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11 194 Harmonization Guidelines for Good Clinical Practice. The protocol was developed by the
12
13 195 investigators and it was approved by the Cyprus National Bioethics Committee and was
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15 196 overseen by an independent safety-monitoring committee evaluating the safety and over-all
16
17 197 benefit-risk profiles. The adherence of care providers with the protocol was assessed by an
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19 198 external committee assigned by the funder of the project through reviews of case report
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21 199 forms. All patients gave written informed consent at the time of enrolment.
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27 201 **Randomization and masking**

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29 202 Patients were randomly assigned to four intervention groups in a 1:1:1:1 ratio stratified by
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31 203 gender (women to men, 3:1). Randomization was facilitated by a lottery-type pool of
32
33 204 numbered balls. Patients were randomly assigned to treatment in blocks of four by flipping a
34
35 205 coin as follows: for the first two drawn balls heads stratified them to the groups A/B and tails
36
37 206 stratified them to the groups C/D. The other two balls were stratified accordingly. A second
38
39 207 toss of the coin assigned the two patients to group A (head)/B (tail) or group C (head)/D
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41 208 (tail). The randomization scheme was generated, performed and securely stored by Helix
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43 209 Incubator Organization of Nicosia University (HIONU).
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49 211 The interventions had identical appearance and smell in dark bottles (15 daily-dose
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51 212 portions/bottle) under nitrogen bed and labeled by HIONU with code numbers, unidentifiable
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53 213 for both patients and investigators. Study data were collected by the investigators and saved
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55 214 by the HIONU that also held the blinded codes of the study. All study personnel involved in
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3 215 the conduct of the study were blinded throughout the study. Treating/examining physician,
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5 216 other investigators, pharmacist, neuroradiologist and patients were masked to treatment
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7 217 allocation.
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11 219 **Procedures and end points**

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14 220 The specific omega-3 (re-esterified glycerides) and omega-6 (glycerides) raw materials were
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16 221 purchased according to the required interventions' PUFA-fraction specification (molecular
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18 222 structure, quantity/ratio and quality) with vitamin E (alpha-tocopherol) used as antioxidant
19
20 223 stabilizer by the supplier. The vitamins and masking aroma were purchased separately. The
21
22 224 mixing of fractions to the final required intervention-composition specification was always
23
24 225 performed by the same team of scientists under the supervision of the involved medical
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26 226 biochemist and lipidology specialist, under appropriate conditions every six months.
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28 227 Interventions were stored refrigerated in dark until use. See Table 1 and Supplementary
29
30 228 Information Methods 1 and 2 for intervention specification detailed description and
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32 229 study/intervention rational.
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38 231 Participants were randomly assigned to receive a daily dose of a mixture of EPA (1,650mg) /
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40 232 DHA (4,650mg) / GLA (2,000mg) / LA (3,850mg) / total other omega-3 (600mg) / total
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42 233 MUFA (1,714mg) + total SFA (18:0 160mg, 16:0 650mg) / vitamin A (0.6mg) / vitamin E
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44 234 (22mg) (intervention A, group A); or composed mixture of pure γ -tocopherol (760mg)
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46 235 dispersed in pure virgin olive oil (16,137 mg) as delivery vehicle (intervention C, group C);
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48 236 or a mixture of intervention formula A with intervention C without the pure virgin olive oil
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50 237 (intervention B, named PLP10, group B); or placebo composed of pure virgin olive oil
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52 238 (16,930mg) (intervention D, group D) (Table 1). Citrus-aroma was used as masking agent of
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54 239 the taste and odor and added in each one of the intervention for a total of 19.5ml dosage of
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3 240 solution per day. The institution's pharmacist was responsible for the appropriate storage and
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5 241 handling of the interventions to the individual participants. The interventions were taken
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7 242 orally once daily 30 minutes before dinner by a dosage calibrated cup for 30 months. The
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9 243 ingredients, ratio and dose have been selected based on their biophysical interrelation to the
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11 244 total known multiple MS causing factors, their biochemical importance and the role expected
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13 245 to play in the normalisation and treatment of the involved complex network of events in the
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15 246 disease pathophysiology. Moreover, the high intake dosage was used to overcome any
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17 247 abnormal dietary accumulation of related agents as a result of patients' food intake habits,
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19 248 irrespective of geographical origin, in relation to the daily consumption ratio of the total fatty
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21 249 acid intake; in order to end-up with omega-3 to omega-6 PUFA indicated physiological body
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23 250 ratio composition of 1:1 wt/wt.
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29 252 The period beginning from July 1st 2007 (enrolment) until December 31st 2007 (entry
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31 253 baseline) was used for normalization period. This six-month normalization period would
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33 254 allow the interventions' agents to exert their beneficial effect (for the
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35 255 incorporation/normalization of cell membranes by oral PUFA, since they need four to six
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37 256 months to exert pivotal action on immune and neural cells, correction of antioxidant
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39 257 deficiency and body PUFA, and optimal normalization of EPA and DHA levels/ratio).⁴¹⁻⁴³
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43 258 The study was completed on December 31st 2009 and the recording of relapses continued
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45 259 until December 31st 2010. More clearly the study included the "normalization period" (July
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47 260 1st 2007 to Dec 31st 2007), the "on treatment" period (Jan 1st 2008 to Dec 31st 2009) and the
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49 261 12-month "extended period" (Jan 1st 2010– Dec 31st 2010).
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54 263 Depending on their clinical status and in accordance with the ethical issues governing clinical
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56 264 trials participants continued receiving the indicative regular available treatments, according to
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3 265 international guidelines with persistent evaluation of any side-effects and adverse events.

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5 266 The study was designed to end 30 months after enrolment and clinical assessments were

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7 267 scheduled at entry baseline, 3, 9, 15, 21 and 24 months on-treatment. Patients were also

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9 268 clinically examined by the treating neurologist within 48 hours after the onset of new or

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11 269 recurrent neurologic symptoms.

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16 271 The primary end point was the ARR at two years. A relapse was defined as new or recurrent

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18 272 neurologic symptoms not associated with fever or infection that lasted for at least 24 hours

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20 273 and accompanied by new neurologic signs. Relapses were treated with methyl-prednisolone

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22 274 at a dose of 1g intravenous per day, for three days followed by prednisone orally at a dose of

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24 275 1mg/kg of weight per day on a tapering scheme for three weeks. The secondary end point at

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26 276 two years was the time to confirmed disability progression, defined as an increase of 1.0 or

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28 277 more on EDSS, confirmed after six months (progression could not be confirmed during a

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30 278 relapse). The final EDSS score was confirmed six months after the end of the study. A post-

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32 279 hoc analysis was performed assessing the proportion of patients free from new or enlarging

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34 280 T2 lesions on brain MRI scans at the end of the study for the per-protocol participants of the

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36 281 group receiving the highest effective intervention versus placebo. Comparison was made only

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38 282 versus the available archival MRI scans up to three months before the enrolment date. MRI

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40 283 scans were performed and blindly analyzed at an MRI evaluation centre. The patients

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42 284 continued to be followed for additional 12 months after completion of the trial and relapses

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44 285 were recorded. Finally, patients were strongly encouraged to remain in the study for follow-

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46 286 up assessments even if they had discontinued the study drug.

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53 288 Blood samples were collected from all randomized patients at the time of enrolment, at every

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55 289 scheduled clinical assessment and during relapses. To check individual compliance with

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3 290 intake, the fatty acids composition of patients' red blood cells' membranes was determined,
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5 291 by gas chromatography, according to a standard protocol. The fatty acid analyses were
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7 292 performed after study termination and thus did not influence the blinding.
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11 294 The involved neurologist was experienced with more than 20 years in practice and trained to
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13 295 standardise EDSS scoring procedures, examined patients, made all medical decisions,
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15 296 determined the EDSS score and reviewed adverse or side-effects. The medical biochemist,
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17 297 specialist on lipidology and immunology and the registered clinical dietitian, members of the
18
19 298 investigator team were experienced with more than 25 years in practice. Patients were able to
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21 299 contact the neurologist at any time if there was any adverse event, side-effect or allergic
22
23 300 reaction. The study drug was not suspected to have any clinical or laboratory adverse effects
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25 301 different from placebo that could disturb the double-blind nature of the trial. Therefore, the
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27 302 same study-neurologist functioned as both the treating and evaluating physician.
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34 304 Safety measures were assessed from the time of enrollment until 12 months following study
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36 305 completion. Haematological and biochemical tests were performed at enrolment and at every
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38 306 12 months, including full blood count, renal and liver function tests, proteins, cholesterol,
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40 307 triglycerides, glucose and electrolytes.
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45 309 The whole procedure followed the clinical trial guidelines as required by the USA Food and
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47 310 Drug Administration, European Medicines Agency, and the Committee for Medicinal
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49 311 Products for Human Use.⁴⁴
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54 313 **Statistical analysis**
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3 314 Power calculations could not be done before the study because of the lack of information
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5 315 from previous studies on potential effect sizes. In 2005 the prevalence of MS in Cyprus
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7 316 (600,000 population) was 120/100.000. Based on the aforementioned MS patients' numbers
8
9 317 of our country and the centre of reference, the CING, we were able to enrol the 20% of the
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11 318 total RRMS patients eligible for treatment in the trial. Sample size was strictly based on this
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13 319 subjects' availability parameter and the novelty of the assessed intervention.
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18 321 Baseline characteristics were compared across all intervention groups by ANOVA or
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20 322 Kruskal-Wallis rank test for continuous variables and by mean Fisher's exact test for
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22 323 categorical variables, as appropriate.
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27 325 For the primary outcome, the ARR was analysed in a pair-wise fashion for the active
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29 326 interventions compared to placebo using negative binomial regression models adjusted for
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31 327 number of relapses within two years before baseline, EDSS score at baseline and DMT. The
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33 328 relapse rate was calculated as the total number of relapses divided by the total number of
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35 329 patient-years followed for each treatment group. ARR differences were also calculated
36
37 330 among all comparable parameters and reported as percent difference.
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42 332 For the secondary end-point outcome, the time to disability progression, Kaplan–Meier
43
44 333 curves were constructed. Progression to disability and time thereof was compared in a pair-
45
46 334 wise fashion for the active interventions versus placebo by the log-rank test in the main
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48 335 analysis and by Cox proportional-hazards models with adjustment for baseline EDSS score,
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50 336 age and DMT in the supportive analysis. Each test was performed with a significance level of
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52 337 0.05. Multivariate models considered all variables with $P < 0.1$ on univariate models. There
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54 338 was no overt violation of the proportionality assumption.
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340 Both, per-protocol and intention to treat (ITT) analyses were performed, for different sets of
341 research questions to be answered, and both are reported. Missing data of the five lost to
342 follow patients were imputed by use of the last-observation-carried-forward (LOCF)
343 approach. Due to the proof-of-concept design of the study, the considerable non-adherence
344 rate (49%) and the interpretation issues caused thereof regarding the ITT analysis, the per-
345 protocol analysis considered being more informative and appropriate method approach to
346 answer the research addressed questions of efficacy of the interventions when subjects were
347 continuously following the protocol. All statistical analyses were well defined a priori. All
348 analyses were performed with STATA SE 10.0 (College Station, TX, USA). P-values are
349 two-tailed.

350

351 **Role of the funding source**

352 The funders had no role in study design, data collection and analysis, decision to publish, or
353 preparation of the manuscript. All members of the writing group had full access to all study
354 data and contributed to its interpretation and prepared, reviewed, and approved the
355 manuscript for submission. All authors had final responsibility for the decision to submit the
356 paper for publication.

357

358 **Results**

359 **Study population**

360 From July 2007 through December 2010 (including the 12-month extended period), a total of
361 80 MS patients were randomly assigned to a study group at the CING (tertiary neurological
362 center).

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3 364 Among the 80 patients, 20 patients were randomly assigned to each of the three groups to
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5 365 receive the interventions and 20 to receive placebo (Fig 2). Baseline characteristics of both
6
7 366 the ITT and the per-protocol populations were similar across groups (Table 2A and 2B).
8
9 367 Total drop-out patients completed follow-up until study completion and were included in the
10
11 368 ITT analyses (Table 4). Five patients were totally lost to follow before their first scheduled
12
13 369 visit and two patients dropped-out before their first scheduled visit progressed to secondary
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15 370 progressive MS. Fifteen patients dropped-out without successfully completing the
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17 371 “normalization” period including five pregnancies. Another 17 patients dropped-out early
18
19 372 after entry baseline. Seven patients that dropped out were given monoclonal antibody
20
21 373 treatment (natalizumab). Overall, a total of 41 (51%) patients completed the 42-month study
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23 374 (July 2007 through December 31st 2010, including the 12-month extended period) where one
24
25 375 patient from group A and two from the placebo group transferred on natalizumab, and 39
26
27 376 (49%) patients either withdrew (drop-out) or lost to follow. Reasons for study-interventions
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29 377 discontinuation are listed in Figure 2.
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36 379 **Efficacy**

38 380 **Relapses**

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40 381 As a proof-of-concept trial we primarily needed to answer whether the interventions were
41
42 382 effective for those MS patients who adhere to the assigned treatment, the per-protocol
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44 383 analysis.⁴⁵ For the sake of methodological comprehensiveness we also present the ITT
45
46 384 analysis as a secondary analysis, to answer a different question, complementary to our core
47
48 385 hypothesis; like what happened to MS patients who were placed on the interventions (the
49
50 386 effect of assignment).⁴⁵ Otherwise, as a result of a high drop-out rate, an ITT analysis will not
51
52 387 likely be able to show the superiority of an intervention even if it is effective.⁴⁵ In any
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54 388 instance, the proper approach of evaluating a study data is to understand what question
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3 389 prompted the research and assure that the analysis is appropriate for providing the answer
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5 390 whatever it is called. Both analyses can be performed for a study, using the results from the
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7 391 different analyses to answer different research questions.⁴⁵ These interventions are original,
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9 392 composed by a different treatment rational, the SM, never tested before and the important
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11 393 main concern was to evaluate their efficacy and safety based on the per-protocol treated MS
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13 394 patients, without any peripheral noise. The question that had to be answered was: “what
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15 395 happens to the patients that are placed and stick on the specific treatment”.

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21 397 Regarding the per-protocol analysis, during the first year of treatment, the ARR was 0.80,
22
23 398 0.40, 0.78 and 0.83 for the four intervention groups respectively. During the second year, the
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25 399 ARR was 0.90, 0.40, 0.67 and 1.25 for the four intervention groups respectively. Overall, for
26
27 400 the two-year primary end point, 8 relapses were recorded for the 10 patients in PLP10 group
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29 401 (0.40 ARR) versus 25 relapses for the 12 patients in placebo (1.04 ARR), a 64% adjusted
30
31 402 relative rate reduction (RRR) for the PLP10 group (RRR 0.36, 95% confidence interval (CI)
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33 403 0.15 to 0.87, $p=0.024$) (Tables 3A, 5 and Fig 3A and 3C). Excluding patients on monoclonal
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35 404 antibody (natalizumab) treatment, the observed adjusted RRR became stronger (72%) over
36
37 405 the two years (RRR 0.28, 95% CI 0.10 to 0.79, $p=0.016$, Tables 3B and 5). Pair-wise
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39 406 comparisons for the other two groups against placebo did not yield statistically significant
40
41 407 results (Tables 3A, 3B). The proportion of patients with ≤ 1 relapse for the two years on-study
42
43 408 was higher in the PLP10 group than in the placebo group (90% versus 42%, $p=0.030$, Table
44
45 409 5). Seeking to investigate further the observed difference, we compared the relapse rate
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47 410 during the 24 months before entry to the study to the 24 months on-treatment for each
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49 411 intervention group. We observed a statistically significant relative reduction in the ARR
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51 412 (70%) only in the PLP10 group (RRR 0.30; 95% CI 0.14 to 0.65, $p=0.003$, Table 3A);
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53 413 within-group comparisons for the three other groups ARR reduction was not significant and
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3 414 remained not significant when natalizumab treated patients were further excluded from the
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5 415 analysis. The effect of PLP10 through time at different time-windows versus placebo for all-
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7 416 time on-study patients is shown in Figures 3A to 3D. The ARR analysis, within time-
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9 417 windows, was not an assigned endpoint, but it could help in the process of evaluating parallel
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11 418 information as the time needed for a specific treatment intervention activity to be evident, as
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13 419 well as the efficacy profile through time. PLP10 reached maximum effect within a year on-
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15 420 treatment (counting from the entry baseline) and remained stable at an ARR of 0.4,
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17 421 displaying a steadily reduced ARR with long free-relapse time-windows. These group B
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19 422 characteristics are considered important parameters of a successful MS treatment where the
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21 423 rule than the exception is the heterogeneity among patients' disease evolution. Specifically,
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23 424 Figure 3D demonstrates the dispersion of relapses throughout the 2-year period of all-time
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25 425 on-study (excluding patients on natalizumab) of PLP10 (n=10) versus placebo (n=10).
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29 426 Placebo, in line with the existing knowledge of how relapse history works in relation to future
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31 427 relapses on MS patients (contagion phenomenon) indicates the expected linearly increased
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33 428 trend of the relapse incidence.⁴⁶ The same phenomenon was true for the groups A and C.
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36 429 Finally, during the 12 month post-study extended period (January 1st 2010 to December 31st
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38 430 2010) all-time on-study patients that received PLP10, showed persistent benefit in the ARR
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40 431 compared to placebo (six relapses for the 10 subjects within PLP10, 0.6 ARR versus 19 for
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42 432 the 12 subjects within placebo group, 1.58 ARR) indicating a statistically significant 62%
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44 433 adjusted relative rate reduction in the ARR for the PLP10 group (RRR 0.38, 95% CI 0.12 to
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46 434 0.99, p=0.046).

435

436 Regarding the ITT analysis, within PLP10 group, none of the nine drop-out patients changed
437 to natalizumab and two out of nine discontinued because of pregnancy, whereas two out of
438 seven drop-out patients from the placebo group changed to natalizumab (a total of four

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3 439 patients within the placebo arm population were on natalizumab, including the two patients
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5 440 that transferred while all-time on-study versus none within PLP10 group (Supplementary
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7 441 Information Fig 1). As reported, natalizumab reduces ARR by 68%, decreases the possibility
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9 442 of disability progression by 43% with 57% of patients free of new or enlarging T2 lesions on
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11 443 MRI scans compared to 15% on placebo.⁴⁷ The relapses of the drop-out patients are reported
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13 444 in Table 4A. As expected no statistically significant differences in the ARR were calculated
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15 445 for the comparison of any group versus placebo for the 24 months on-treatment, with a 0.75
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17 446 ARR within PLP10 (30 relapses) and 1.03 ARR within placebo (41 relapses) group, a 27%
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19 447 ARR reduction (Table 4B). Interestingly, despite the high non-adherence rate, there was a
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21 448 statistically significant difference for the comparison of the ARR in the 24 months before
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23 449 entry baseline to the 24 months on-treatment for the PLP10 group (RRR 0.45, 95% CI 0.26 to
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25 450 0.78, p=0.005).

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452 **Disability progression**

453 Regarding the per-protocol analysis, at two years, the time to disability progression, with
454 confirmation after six months (secondary end-point) was significantly longer only with
455 PLP10. The cumulative probability of disability progression was 10% in the PLP10 group
456 and 58% in the placebo group (p=0.019) (Supplementary Information Fig 2). After excluding
457 patients on natalizumab, there was an increased statistically significant difference between
458 the PLP10 and the placebo group for the same analysis (p=0.006) (Fig 4A). At two years, the
459 cumulative probability of progression was 10% in the PLP10 group and 70% in the placebo
460 group, which represents a decrease of 60 percentage points or a relative 86% decrease in the
461 risk of sustained progression of disability within PLP10 group (adjusted hazard ratio, 0.11;
462 95% CI 0.01 to 0.97, p=0.047). One versus seven out of ten patients progressed to confirmed
463 disability in the PLP10 and the placebo groups respectively when patients on natalizumab

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3 464 were excluded. No statistically significant difference was observed for any comparison of the
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5 465 other two groups compared to placebo (Fig 4A and Supplementary Information Fig 2).
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9 467 Regarding the ITT analysis, at two years, the cumulative probability of progression was 10%
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11 468 in the PLP10 group and 35% in the placebo group ($p=0.052$, a trend for an effect), which
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13 469 represents a decrease of 25 percentage points or a relative 71% decrease of the PLP10 for the
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15 470 risk of sustained progression of disability (adjusted hazard ratio 0.22, 95% CI 0.04 to 1.07,
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17 471 $p=0.06$) (Fig 4B). Two versus seven out of the total randomized patients progressed to
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19 472 confirmed disability in the PLP10 and the placebo groups respectively. No significant
20
21 473 differences were observed for groups A or C against placebo (Fig 4B). The mean change in
22
23 474 Expanded Disability Status Scale (EDSS) score as a function of visit number is shown in
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25 475 Figure 5.
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31 477 **MRI**

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33 478 Over two years, the MRI results support the overall conclusion from the study that PLP10 has
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35 479 a positive effect on disease activity since only 29% from the PLP10 group as opposed to 67%
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37 480 from the placebo group developed new or enlarging T2 lesions (57% relative risk reduction).
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39 481 Excluding patients on natalizumab there is an increased relative risk reduction (64%) between
40
41 482 PLP10 as opposed to placebo with 29% of patients on PLP10 and 80% on placebo with
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43 483 development of new or enlarging T2 lesions (Table 5).
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49 485 **Safety**

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51 486 Over the course of the 30 month study no significant adverse events were reported from any
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53 487 group. According to a questioner procedure the only aetiology for drop-outs was the
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55 488 palatability and smell of the formula preparations. Nausea was reported by two patients. No
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3 489 abnormal values observed on any of the biochemical and haematological blood tests. No
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5 490 allergic reactions reported.
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9 10 492 **Discussion**

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12 493 In this proof-of-concept clinical trial assessing the safety and efficacy of three cocktail
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14 494 intervention formulas in RRMS, we observed a significant benefit for the novel PLP10
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16 495 intervention compared to placebo for both the ARR and the progression to disability. Our
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18 496 results include analyses pertaining to a total of 42 months study collected data, including the
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20 497 12-month, free of intervention treatment, extension period. We focused on the per-protocol
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22 498 data analysis since it is the appropriate method to best provide the answer to the proof-of-
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24 499 concept trial-addressed question. The high drop-out rate was solely the result of formulas
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26 500 palatability, a common phenomenon in trials using oily interventions where a lot of patients
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28 501 tend to drop-out soon after first dosage. We thus present our main per-protocol analysis, as
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30 502 well as a subgroup analysis excluding patients on natalizumab. We have found a statistically
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32 503 significant reduction in the ARR and the disability progression comparing not only patients
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34 504 on PLP10 versus placebo but also comparing the ARR of the PLP10 patients in the 24-month
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36 505 period prior to the study to the ARR of the 24 months on-study; the observed differences
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38 506 became larger when patients that received natalizumab (the most potent disease modifier)
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40 507 were excluded. The ARR decreased within a year on PLP10 and significantly remained stable
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42 508 until study completion. Statistically significant difference of ARR between patients on PLP10
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44 509 versus placebo continued for the additional 12 month extended period (persistent effect)
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46 510 without significant difference on DMT. These clinical findings are supported by the results
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48 511 regarding the MRI analysis where the proportion of patients free from new or enlarging brain
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50 512 T2 lesions was also higher in PLP10 group versus placebo. The persistent effect within the
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52 513 extended period it is considered of major importance and supportive of the results since it is
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3 514 in agreement with the very long washouts, reported necessary, for omega-3 fatty acids and
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5 515 especially DHA to return towards pretreatment values within the fatty acids of plasma,
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7 516 platelets, monocytes and red blood cells.⁴² This study also provides important 30-month,
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9 517 placebo-controlled information about the safety of PLP10, A and C interventions, where no
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11 518 any adverse or severe side effects have been reported.
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16 520 As medications used to treat MS become increasingly highly specific and potent, attention to
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18 521 safety is paramount. Current available treatments are products of reductionism, partially
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20 522 effective, associated with severe side effects without (re)myelinating or neuroprotective
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22 523 abilities. Interferons and glatiramer acetate, the most widely used first-line MS drugs
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24 524 available today, are associated with the least severe side-effects among MS therapies but they
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26 525 are reported with only 29-33% ARR reduction and with no significant effects on the
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28 526 progression of disability. Natalizumab as previously discussed and Fingolimod with 54%
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30 527 ARR reduction (without significant benefit on the progression of disability) are second-line
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32 528 drugs associated with severe side-effects.^{47, 48}
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38 530 No existing MS treatment has ever been designed as a result of SM concept approach or with
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40 531 a potential to effectively stimulate intrinsic remyelinating and neuroprotecting mechanisms or
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42 532 exert such an action. Now we propose that a holistic SM model approach has to be applied by
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44 533 synchronized action on all involved perturbed mechanisms. PLP10 has innovative
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46 534 characteristics like no any other intervention or medication tried before for MS treatment,
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48 535 with unique efficacy abilities through different mechanisms of action, probably by the
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50 536 synergistic effect of its constituent ingredients. PLP10 has all the characteristics of a medical
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52 537 food with the action to feed a normal metabolic process by supplying nutritional structural
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54 538 membrane precursors, building blocks, and vitamins from dietary sources that enhance
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3 539 remyelination and neuroprotection and simultaneously promote normalization of all cellular
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5 540 membranes lipid content. The intention is to normalize the specific nutritional requirements
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7 541 of the MS patients.
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12 543 Different factors and molecular entities appear to be part of the possible aetiology for MS
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14 544 with specific PUFA and antioxidants found to be key substances related to all known
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16 545 pathogenic and recovery mechanisms. But, it is well established that MS patients are
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18 546 characterized with significant deficiencies in antioxidant efficiency as well as in PUFA levels
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20 547 in blood and cellular membranes.^{11, 49-51}
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25 549 According to one hypothesis, the change in the ratio of omega-3 to omega-6, due to
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27 550 increasing consumption of omega-6 PUFA, meaning high accumulation of AA, in the
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29 551 Western diet, may be one of the major factors responsible for the increasing incidence of
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31 552 inflammatory diseases relative to populations. In the Western diet, the ratio of omega-3 to
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33 553 omega-6 is about 1:20–30; in populations that consume fish-based diets, the ratio is about
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35 554 1:1–2.^{52, 53} The intervention daily dose was aiming and believed to be high enough to
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37 555 restore/amplify body efficient antioxidant activity and ensure cellular membranes lipid profile
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39 556 normalization (PUFA content) and simultaneously potentiate involvement of the ingredients
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41 557 in the anti-inflammatory and recovery mechanisms. Diet fatty acid molecules need about six
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43 558 months period to exert their beneficial effect and this essential parameter was for the first
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45 559 time under consideration in our study design (normalization period).⁴² This chronotherapy
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47 560 parameter it is of major importance in line with the SM treatment philosophy and if it is not
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49 561 included in the trial design the possibility of misleading result evaluation greatly increases. In
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51 562 fact, considering that omega-3 supplementation can release and replace excess AA within the
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3 563 cellular membranes, we can speculate that an increased inflammatory activity can possibly
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5 564 result during the first six months of supplementation (during normalization period).
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10 566 The maintenance of myelin requires continued turnover of its components throughout life.^{54,55}

11 567 In addition to the EPA, DHA, LA, and GLA, PLP10 was containing limited quantities of

12 568 other structural PUFA, specific MUFA (mostly oleic acid) and SFA (palmitic and stearic

13 569 acid), specifically to provide a direct source for neuronal cell membranes rehabilitation and

14 570 for (re)myelination and neuroprotection since they are all major components, precursors and

15 571 building blocks of any new physiological myelin and cellular membranes in general.

16 572 Assembly of the correct molecules into myelin membrane may be especially critical during

17 573 active synthesis. Possibly, if critical constituents aren't available or are metabolically

18 574 blocked, amyelination, dysmyelination or demyelination may ensue.⁵⁶

19 575

20 576 The well known and established safety of the ingredients used and the protocol guidelines

21 577 were supportive reasons for us to proceed with the clinical study even though with limitation

22 578 on the pre-estimation of required trial sample size as it was discussed in method section. The

23 579 adherence of the subjects is another issue but the duration of the study (42 months) is adding

24 580 power to the results;⁴⁴ having the research questions been consciously and carefully

25 581 approached and answered. Furthermore, the statistical methodologies used along with the

26 582 appropriate adjustments, broadly accepted for MS clinical trials, power the findings, results,

27 583 and significance. The baseline characteristics of the treatment arms could possibly be

28 584 considered indicative of four very active groups of patients but that was the result of the

29 585 limited number of RRMS population eligible for the study within Cyprus. On the other hand

30 586 the balanced baseline characteristics without statistical differences, the statistical adjustments

31 587 (for all important baseline parameters i.e. relapses, EDSS, age and DMT) and the

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3 588 randomization within four different groups are the safety valves against data
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5 589 misinterpretation. Yet, in small randomized control trials with a high drop-out rate, the per-
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7 590 protocol analysis could be affected by the characteristics of the patients dropping out. In
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9 591 order to safeguard our findings in the best possible way under the circumstances, we
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11 592 proceeded to adjusting for confounders. Moreover, we cannot discard our finding as a false
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13 593 positive, given that this is a randomized, double-blind, placebo-controlled clinical trial and,
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15 594 despite its small sample size, represents a piece of evidence that only a larger randomized
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17 595 controlled trial can replicate or refute. It is possible to question why DMTs efficacy cannot
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19 596 be emerged out of the data analysis, of the four treatment arms, and in accordance to their
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21 597 published values. We believe that the limited efficacy of the DMTs, the sample size and the
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23 598 statistical adjustments were strong limiting determining factors for such an indication to be
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25 599 countable. An additional argument is that the efficacy reported for the analysis of pre-
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27 600 treatment (24 months before entry baseline) versus on-trial ARR could be considered as
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29 601 potentially biased due to differences of how relapses were defined during the course of a
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31 602 study compared to pre-treatment period; or due to regression to the mean or placebo effect.
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33 603 This analysis was performed as an additional exploratory analysis that we were able to do due
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35 604 to the availability of data. The relapses of the two pre-treatment years were drawn out of the
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37 605 patients' archival records by the same treating neurologist involved in the study (MP), and
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39 606 according to the patients' hospitalization date for receiving intravenous methyl-prednisolone.
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41 607 This analysis was not used as a primary or a secondary end-point under investigation
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43 608 although it is usually reported by many clinical studies. As a matter of fact many early phase
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45 609 trials are based only on such an analysis (before versus after treatment results). In almost all
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47 610 MS trials the number of relapses within the two years before baseline is a factor under
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49 611 adjustment for the statistical analyses.⁴⁸ The inclusion of the post-hoc MRI analysis is another
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51 612 limiting factor that needs attention since it was used as an additional aside exploratory
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3 613 approach (due to study budget limitations it was not possible to be used as a formal
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5 614 endpoint); but the MRI evaluation was blinded and can be considered as representative of the
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7 615 randomized subjects within the treatment arms. As far as the regression to the mean and the
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9 616 placebo effect concerns we believe that the 6-month normalization period is an accountable
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11 617 and valuable eliminating factor of the possible effect; as well as the presence of four groups,
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13 618 where only the PLP10 treatment arm is associated with statistically significant efficacy versus
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15 619 placebo. It is a placebo-controlled study after all.
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21 621 Our observations are consistent with the idea that simultaneous availability of specific PUFA
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23 622 along with other major membrane and myelin building blocks in combination with specific
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25 623 antioxidants, within optimum quantity, quality, ratio and structural form can possibly result to
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27 624 a more appropriate holistic therapy reducing MS disease activity. This is probably succeeded
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29 625 through synergistic and/or simultaneous effect on the interactions and dynamics of the most
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31 626 probable environmental and biological disease causing factors that induce complex biological
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33 627 network of events for disease pathogenesis and evolution; as well as on the protective and
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35 628 reparative mechanisms. We can additionally speculate that the nature of the intervention
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37 629 formula cannot be prohibitive for its use as preventive regimen and does not preclude
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39 630 probable positive efficacy on the other types of MS, but has to be further investigated. A
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41 631 larger size multicenter clinical trial will better establish PLP10 place in the armamentarium of
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43 632 treatments for MS.
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48
49 634 It is commonly accepted that nutrition is one of the possible environmental factors involved
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51 635 in the pathogenesis of MS, but its role as complementary MS treatment is unclear and largely
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53 636 disregarded.⁵⁷ It is well known that the majority of the patients suffering from MS they do
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55 637 use dietary supplements for a variable length of time and they prefer supplement type of
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3 638 “help” over conventional drugs.⁵⁸ Dietary antioxidants and fatty acids may influence the
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5 639 disease process in MS by reducing immune-mediated inflammation, oxidative stress and
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7 640 excitotoxic damage.¹¹ Present data reveal that healthy dietary molecules have a pleiotropic
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9 641 role and are able to change cell metabolism from anabolism to catabolism and down-regulate
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11 642 inflammation by interacting with enzymes, nuclear receptors and transcriptional factors.⁵⁷
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13
14 643 The present study, for the first time provides strong link evidence between dietary, metabolic,
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16 644 immunological, and neurobiological aspects of MS after three quarters of a century of
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18 645 unsuccessful scientific efforts. This might probably be the beginning of opening new
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21 646 horizons and new avenues in the approach of MS prevention and treatment, and possibly of
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23 647 other multifactorial chronic diseases, including neurodegenerative and autoimmune as well.
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1.
Treatment Arms

A	B (PLP10)	C	Placebo
Intervention: EPA (1,650mg) / DHA (4,650mg) / GLA (2,000mg) / LA (3,850mg) / total other omega-3 (600mg)* / total MUFA** (1,714mg) + total SFA (18:0 160mg, 16:0 650mg) / vitamin A (0.6mg) / vitamin E (22mg)	Intervention: EPA (1,650mg) / DHA (4,650mg) / GLA (2,000mg) / LA (3,850mg) / total other omega-3 (600mg)* / total MUFA** (1,714mg) + total SFA (18:0 160mg, 16:0 650mg) / vitamin A (0.6mg) / vitamin E (22mg) + pure γ -tocopherol (760mg)	Intervention: pure γ -tocopherol (760mg) dispersed in pure virgin olive oil (16,137 mg) as delivery vehicle	Intervention: Olive oil (pure virgin)

* Other omega-3: C18:3n-3 37mg, C18:4n-3 73mg, C20:4n-3 98mg, C22:5n-3 392mg
 ** MUFA: 18:1 1300mg, 20:1 250mg, 22:1 82mg, 24:1 82mg

EPAX1050, EPAX AS, Aalesund, Norway; was used as the source for the omega-3 PUFA, as re-esterified glycerides from fish body oils; Borage seed oil (organic, cold pressed) “Borago officinalis” Goerlich Pharma International GmbH, Edling, Germany, was used as the source for the omega-6 PUFA, MUFA and SFA, as triglycerides. The pure γ -tocopherol was purchased from Tama Biochemical Co. Ltd., Shinjuku-ku Tokyo, Japan; vitamin A as beta-carotene from HealthAid Ltd., Middlesex, United Kingdom and the Citrus aroma from Givaudan Schwaiz AG, Dubendorf, Switzerland.

Table 1. Intervention ingredients per treatment arm. Citrus-aroma was used as masking agent of the taste and odor and added in each one of the intervention for a total of 19.5ml dosage of solution per day.

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2A.					
Characteristics	Group A (n=20)	Group B† (n=20)	Group C (n=20)	Placebo (n=20)	P- value
Sex					
Female - no. (%)	15 (75)	15 (75)	15 (75)	15 (75)	1.000
Age (yr)					
Mean ± Standard Deviation (SD)	38.0±11.9	36.9±8.4	37.7±8.7	38.1±10.9	0.982
Median (Range)	38.0 (22–65)	37.0 (25–61)	36.5 (24–54)	36.0 (21–58)	
Treatment history					
Patients on DMT- no. (%)	11 (55)	9 (45)	12 (60)	10 (50)	0.875
Pre-treatment disease duration (yr)					
Mean ± SD	9.0±7.6	8.6±4.8	8.6±5.3	7.7±5.7	0.909
Median (Range)	7.5 (2–37)	8.0 (2–20)	8.0 (3–24)	6.5 (2–25)	
Pre-treatment relapses‡					
Mean ± SD	2.33±1.68	2.41±1.73	2.31±1.66	2.10±1.32	0.946
Median (Range)	2.0 (1–6)	2.0 (1–7)	2.0 (1–6)	2.0 (1–4)	
ARR	1.17	1.21	1.16	1.05	0.946
Patients -% with ≤1 relapse	40	45	40	35	
Baseline EDSS score‡					
Mean ± SD	2.52±1.23	2.15±1.05	2.42±1.21	2.39± 0.93	0.775
Median (Range)	2.5 (1.0–5.5)	2.0 (1.0–4.0)	2.5 (0.0–5.0)	2.5 (1.0–4.0)	
2B.					
Characteristics	Group A (n=10)	Group B† (n=10)	Group C (n=9)	Placebo (n=12)	P- value
Sex					
Female - no. (%)	5 (50)	7 (70)	6 (66.6)	10 (83.3)	0.419
Age (yr)					
Mean ± SD	36.6±13.5	34.80±5.4	40.9±8.1	39.8±13.2	0.572
Median (Range)	34.5 (22–65)	34.5 (26–43)	40.0 (29–54)	37.5 (21–58)	
Treatment history					
Patients on DMT- no. (%)	6 (60)	4 (40)	6 (67)	6 (50)	0.949
Pre-treatment disease duration (yr)					
Mean ± SD	9.7±10.0	8.3±5.3	11.3±6.1	8.7± 7.1	0.807
Median (Range)	7.5 (2–37)	8.0 (2–20)	8.0 (4–24)	5.5 (2–25)	
Pre-treatment relapses					

Mean ± SD	2.20±1.47	2.70±1.25	1.78±0.66	1.67±1.37	0.241
Median (Range)	2.0 (1 – 6)	2.5 (1 – 4)	2.0 (1 – 3)	1.5 (1 – 4)	
ARR	1.10	1.35	0.89	0.83	
Patients -% with ≤1 relapse	30	20	33	50	
Baseline EDSS score					
Mean ± SD	2.65±1.37	2.40±1.12	2.11±1.02	2.16±0.96	0.698
Median (Range)	3.0 (1.0–5.5)	2.5 (1.0–4.0)	2.0 (1.0–4.0)	2.0 (1.0–3.5)	
† PLP10 group					
‡ Available data at Entry Baseline (n=18 for group A, n=17 for group B, n=19 for group C, n=19 for group D)					

Table 2. The table section 2A reports the demographics and baseline disease characteristics for total randomized population by treatment arm.

The table section 2B reports the demographics and baseline disease characteristics of all-time on-study population by treatment arm. There were no significant between study-group differences at baseline for any characteristic.

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3A.								
Characteristics	Group A (N=10)		Group B† (N=10)		Group C (N=9)		Placebo (N=12)	
End Point	X	Y	X	Y	X	Y	X	Y
Total No. of relapses	22	17	27	8	16	13	20	25
Annual Relapse Rate (ARR)	1.10	0.85	1.35	0.40	0.88	0.72	0.83	1.04
% Reduction Compared to Placebo (primary endpoint) (Ys)¶		-18		-62		-30		N/A
P Value against placebo		0.468		0.024		0.578		
ARR change -% (Y to X)¶		-23		-70		-18		+25
P value against baseline		0.425		0.003		0.578		0.500
X: Total number of relapses of 24 months pre-treatment (baseline) Y: Total number of relapses of 24 months on-treatment ¶ Unadjusted estimate								
3B.								
Excluding patients on natalizumab	Group A (N=9)		Group B† (N=10)		Group C (N=9)		Placebo (N=10)	
End Point	X	Y	X	Y	X	Y	X	Y
Total No. of relapses	16	15	27	8	16	13	13	19
Annual Relapse Rate (ARR)	0.88	0.83	1.35	0.40	0.88	0.72	0.65	0.95
% Reduction Compared to Placebo (primary endpoint) (Ys)¶		-13		-58		-24		N/A
P Value against placebo		0.493		0.016		0.412		
ARR change -% (Y to X)¶		-6		-70		-18		+46
P value against baseline		0.857		0.003		0.578		0.354
X: Total number of relapses of 24 months pre-treatment (baseline) Y: Total number of relapses of 24 months on-treatment † PLP10 group ¶ Unadjusted estimate								
Table 3. The table section 3A reports the two year primary end points of ARR of all-time on-study population by treatment arm and percent difference with placebo. During the 24mo period on-treatment								

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3 the ARR of the group A was 0.85 with 18% decrease compared to placebo (p=0.468), of PLP10 group
4 0.40 with 62% decrease (p=0.024), and of group C 0.72 with 30% decrease (p=0.578) and reports the
5 comparison of the 24mo pre-treatment ARR (baseline ARR) to 24mo on-treatment ARR of all-time on-
6 study population including patients on natalizumab.
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14 The table section 3B reports comparison of the 24mo pre-treatment ARR to 24mo on-treatment ARR of
15 all-time on-study population excluding patients on natalizumab; and the comparison of the ARR during
16 the 24mo period on-treatment (primary end point) between each one of the groups against placebo.
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4A.

Characteristics	Group A (N=8)		Group B† (N=7)		Group C (N=10)		Placebo (N=7)	
	X	Y	X	Y	X	Y	X	Y
No. of Relapses	20	14	14	14	27	26	20	13
Annual Relapse Rate (ARR)	1.25	0.88	1.00	1.00	1.35	1.30	1.42	0.92

X: Total number of relapses of 24 months pre-treatment

Y: Total number of relapses of 24 months on-treatment

4B.

Characteristics	Group A (N=20)		Group B† (N=20)		Group C (N=20)		Placebo (N=20)	
	X	Y	X	Y	X	Y	X	Y
End Point	X	Y	X	Y	X	Y	X	Y
No. of Relapses	45	34	49	30	46	41	43	41
Annual Relapse Rate (ARR)	1.13	0.85	1.23	0.75	1.15	1.03	1.08	1.03
ARR Reduction -% (Y to X)¶	-25		-39		-10		-5	
P value against baseline	0.120		0.005		0.475		0.652	
% Reduction of the ARR Compared to Placebo (Ys)¶	-18		-27		0.0		N/A	
P Value against placebo	0.447		0.121		0.996			

X: Total number of relapses of 24 months pre-treatment (baseline)

Y: Total number of relapses of 24 months on-treatment

¶ Unadjusted estimate

† PLP10 group

Table 4. The table section 4A reports the two year primary end point of relapses based on study design as reported by drop-out patients by Treatment Arm. The most drop-out patients that went on disease modified therapy (DMT) were from the group A and placebo with three and two patients respectively on natalizumab. These parameters justify the decreased number of relapses recorded within group A and placebo drop-outs; and this could affect the ITT analysis in favor of placebo when the total two-year recorded data are used. For PLP10 group, 14 relapses were reported at baseline and remained the same during the two-year study. For placebo, 20 relapses were reported at baseline and decreased to 13 during the two-

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3 year study. This is expected since, for PLP10 group 43% of the drop-outs were under DMT at
4 entry baseline and remained the same until the end of the study with no patient on
5 natalizumab; but 57% of the placebo group drop-outs that were under DMT at entry baseline
6 increased to 86% at the end of the study including two patients on natalizumab.
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14 The table section 4B reports comparison of 24 months pre-treatment ARR (baseline) to 24
15 months on-treatment ARR of total randomized population, by treatment arm. The ARR of
16 PLP10 group was 1.23 at baseline and 0.75 at the end of the study (39% reduction $p=0.005$),
17 and for placebo 1.08 at baseline and 1.03 at the end of the study (5% reduction $p=0.652$). No
18 statistical difference was calculated for the other two treatment arms. During the 24 months
19 on-treatment, PLP10 group presented a 27% reduction of ARR versus placebo ($p=0.121$),
20 with all groups without statistically significant results.
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5.					
Characteristics*	Group A (n=10)	Group B PLP10 (n=10)	Group C (n=9)	Placebo (n=12)	P-value Group B vs. Placebo
Annual relapse rate over 1 year**	0.80	0.40	0.78	0.83	
Total number of relapses**	8	4	7	10	
Primary end points					
Annual relapse rate over 2 years (95% CI)**	0.85	0.40 (0.15-0.87)	0.72	1.04	0.024
Total number of relapses**	17	8	13	25	
Excluding patients on natalizumab					
Annual relapse rate over 2 years (95% CI)	0.83	0.40 (0.10-0.79)	0.72	0.95	0.016
Total number of relapses	15	8	13	19	
Secondary end points					
Cumulative probability of sustained progression increase by 1 point on EDSS confirmed after 6 mo, over 2 years -% **	43	10 (1/10)	24	58 (7/12)	0.019
Excluding patients on natalizumab					
cumulative probability of sustained progression increase by 1 point on EDSS confirmed after 6 mo, over 2 years -%	33	10 (1/10)	24	70 (7/10)	0.006
Exploratory Results					
Patients proportion with ≤1 relapse over 2 years -% **	50 (5/10)	90 (9/10)	56 (5/9)	42 (5/12)	0.030
MRI					
Patients proportion with new or enlarging T2 lesions-% **	----	29 (2/7)	----	67 (4/6)	
Excluding patients on natalizumab					
Patients proportion with no new or enlarging T2 lesions-%	----	29 (2/7)	----	80 (4/5)	
DMT (interferons, glatiramer acetate) and natalizumab					
Patients proportion on DMT and natalizumab at the end of 2 years-% **	80 (8/10)†	60 (6/10)	67 (6/9)	75 (9/12) ‡	0.747
* CI denotes confidence interval.					
** Including patients on natalizumab					
† 1 out of 10 on natalizumab					
‡ 2 out of 12 on natalizumab					

Table 5. Clinical end points, according to study group for all-time on-study population.

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29 751 under a USA provisional patent; Application Number 61469081.
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38 755 All authors have completed the Unified Competing Interest form at
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Article Summary

Article focus:

- The increasing prevalence of Multiple Sclerosis (MS), the limited efficacy and the side effects of the existing treatments urge the clinical need for the development of new, innovative, more effective, safe, and preventive treatment strategies.
- For the first time we propose three original nutraceutical treatment intervention-cocktails, formulated based on systems medicine through nutritional systems biology rational; with PLP10 representing the complete composition of the formulation and the other two treatments represent partial formulations.
- We have studied the proposed interventions on 80 relapsing remitting MS patients (four groups of 20 each) for a total of 42 months, in a randomized, double-blind, placebo-controlled, phase II proof-of-concept clinical trial.

Key messages:

- The per-protocol data analyses indicated that PLP10 is associated with significant efficacy versus placebo on both reducing the annualized relapse rate and disease progression without any adverse or severe side effects.
- PLP10 has probably the ability to interfere with the total known repertoire of mechanisms involved in MS pathogenesis as well as with the recovery mechanisms, neuroprotection and remyelination.; it is potentially able to manipulate global perturbed networks of the disease causing factors rather than focusing only on unique failing components.
- This proof-of-concept clinical study might be indicative and the beginning of new avenues in MS prevention and treatment and of new epoch of novel drug development with dynamic therapeutic potential for chronic complex multifactorial diseases.

Strengths and limitations of this study:

- The strength of this study is the systems medicine therapeutic approach, the length of the study the inclusion of the six months normalization (chronotherapy) period, and the study protocol following all indicated appropriate guidelines with definite inclusion/exclusion criteria and primary/secondary end points.
- The sample size and the high rate of drop-outs (palatability of the formula) are the limitations associated with the present study.
- A phase III multi-center clinical trial will establish the present intervention regime among the best choices among neurodegenerative diseases prophylaxis and MS treatment drugs faretra.

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3 919 **Figure legends**
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6 920 **Figure 1.** Omega-6 and omega-3 PUFA, their respective metabolic derivatives and their
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8 921 possible effects on inflammation.

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11 922 After consumption, the PUFAs are metabolized via several pathways (not shown) to active
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13 923 compounds that mediate inflammation and products that promote resolution of inflammation.

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17 924 Abbreviations: PL, phospholipid; IFN- γ , interferon γ ; IL-2, interleukin 2; NF κ B, nuclear
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19 925 factor kappa B; PGE₂, prostaglandin E₂; PPAR γ , peroxisome proliferator-activated receptor
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21 926 γ ; PUFAs, polyunsaturated fatty acids; TGF β , transforming growth factor β ; TNF, tumor
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23 927 necrosis factor; COX, cyclooxygenase; hydroxyeicosatetraenoic acid; HPETE,
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25 928 hydroperoxyeicosatetraenoic acid; LOX, lipoxygenase; LT, leukotriene; PG, prostaglandin;
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27 929 TX, thromboxane; RXR- γ , retinoid X receptor-gamma; Nrf2, nuclear respiratory factor;
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29 930 MMP, metalloproteinase.
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34 931 **Figure 2.** Study Flowchart

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37 932 **Figure 3.** Panel A demonstrates the ARR of all-time on-study patients during the 24mo pre-
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39 933 treatment (baseline ARR) and at different on-study intervals (6, 12, 18, 24 mo) per treatment
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41 934 arm. **

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44 935 Panel B demonstrates the ARR of all-time on-study population between 0-6, 6-12, 6-18, and
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46 936 6-24 mo period intervals, of PLP10 vs. placebo group. **

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49 937 Panel C demonstrates the ARR of all-time on-study population of PLP10 vs. placebo group at
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51 938 baseline, during 1st year, and during the 2-year on-treatment. **

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55 939 Panel D demonstrates the dispersion of relapses throughout the 2-year period of all-time on-
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57 940 study (excluding patients on natalizumab) of PLP10 (n=10) vs. placebo (n=10). Placebo
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3 941 shows an irregular dispersion of relapses in relation to PLP10 group with linear increasing
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5 942 trend while PLP10 shows a stabilized linear trend. By using the per-protocol model where
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7 943 patients on natalizumab were excluded, we could compare the number of relapses on a same
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9 944 number of patients.

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13 945 ** Including the patients on natalizumab.

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16 946 **Figure 4.** Panel 4A demonstrates the Kaplan–Meier plot of the time to sustained progression
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18 947 of disability among all-time on-study patients, excluding patients on natalizumab, receiving
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20 948 intervention A, PLP10 and C as compared with placebo. PLP10 reduced the risk of sustained
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22 949 progression of disability by 86% over two years (p=0.006). Intervention formula A reduced
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24 950 the risk of sustained progression of disability by 53% (p=0.266) and intervention formula C
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26 951 by 67% (p=0.061).

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30 952 Panel 4B demonstrates the Kaplan–Meier plot of the time to sustained progression of
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32 953 disability among ITT population receiving intervention A, PLP10 and C as compared with
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34 954 placebo. PLP10 reduced the risk of sustained progression of disability by 71% over two years
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36 955 (p=0.052, trend). Intervention formula A reduced the risk of sustained progression of
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38 956 disability by 22% (p=0.727) and intervention formula C by 40% (p=0.447).

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41 957 **Figure 5.** Mean change in expanded disability status scale score as a function of visit
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43 958 number. Values are expressed as mean ± s.e.m.

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1 **A novel oral nutraceutical formula (PLP10) for the**
2 **treatment of relapsing remitting multiple sclerosis: a**
3 **randomized, double-blind, placebo-controlled proof-of-**
4 **concept clinical trial**

5 **Marios C. Pantzaris, George N. Loukaides, Evangelia E. Ntzani, Ioannis S.**
6 **Patrikios**

7 The Cyprus Institute of Neurology and Genetics (CING) 1683 Nicosia, Cyprus Marios C.
8 Pantzaris Senior Consultant Neurologist Neurology Clinic C and PALUPA Medical Ltd., The
9 Cyprus Institute of Neurology and Genetics (CING) 1683 Nicosia, Cyprus George N.
10 Loukaides Clinical Dietitian/Nutritionist Neurology Clinic and PALUPA Medical Ltd.,
11 University of Ioannina School of Medicine (UISM) 45110 Ioannina, Greece Evangelia E.
12 Ntzani Assistant Professor Clinical and Molecular Epidemiology Unit, Department of
13 Hygiene and Epidemiology, PALUPA Medical Ltd at The Cyprus Institute of Neurology and
14 Genetics (CING) 1683 Nicosia, Cyprus Ioannis S. Patrikios Senior Research Scientist,
15 visiting Professor at European University Cyprus Nicosia, Cyprus, Department of Health and
16 Science. Correspondence should be addresses to e-mail: I.Patrikios@euc.ac.cy or
17 pantzari@cing.ac.cy

18
19 **Correspondence to:**

20 **Ioannis Patrikios**

21 The Cyprus Institute of Neurology and Genetics (CING)
22 Neurology Clinic C (PALUPA Medical),

1
2
3 23 6 International Airport Av.
4
5 24 P.O.Box 23462, 1683 Ayios Dometios. Nicosia, Cyprus
6
7 25 Tel: +357 22 358 600, +357 99 097 856;
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10 26 i.patrikios@euc.ac.cy
11
12 27 patrikiosioannis@gmail.com
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16 29 **AND**
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22 31 **Marios Pantzaris**

23
24 32 The Cyprus Institute of Neurology and Genetics (CING)
25
26 33 Neurology Clinic C (PALUPA Medical),
27
28 34 6 International Airport Av.
29
30 35 P.O.Box 23462, 1683 Ayios Dometios, Nicosia Cyprus
31
32 36 Tel: +357 22 358 600;
33
34 37 pantzari@cing.ac.cy
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39 39 **Keywords:** antioxidant vitamins, polyunsaturated fatty acids, multiple sclerosis, systems
40 40 medicine, randomized clinical trial.
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46 42 **Word Count: 6415**
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3 **Abstract**
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5 **Objective** To assess whether our three novel interventions, formulated based on systems
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Design 30-month randomized double-blind, placebo-controlled, parallel design, phase II proof-of-concept clinical study.

Settings Cyprus Institute of Neurology and Genetics (CING)

Participants and Interventions 80 subjects were randomized into four groups of 20. The first intervention (A) was composed of omega-3 and omega-6 polyunsaturated fatty acids at 1:1 wt/wt. Specifically, the omega-3 fatty acids were docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) at 3:1 wt/wt and the omega-6 fatty acids were linoleic acid (LA) and gamma (γ)-linolenic acid (GLA) at 2:1 wt/wt. This intervention also included minor quantities of other specific polyunsaturated, monounsaturated and specific saturated fatty acids as well as vitamin A and vitamin E (alpha-tocopherol). The third intervention (C) was γ -tocopherol alone. The second intervention (PLP10) was a combination of A and C. A fourth group of 20 received a vehicle placebo. The interventions were administered per os once daily.

Main outcome measures The primary endpoint was the annualized relapse rate (ARR) of the three interventions versus placebo at two years. The secondary end point was the time to confirmed disability progression at two years.

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2
3 72 **Results** The per-protocol, proof-of-concept, analysis demonstrated a 64% adjusted relative
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5 73 reduction in ARR at two years for PLP10 versus placebo (P=0.024). Regarding the secondary
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7 74 endpoint, a relative reduction of 86% in the risk of sustained progression of disability was
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10 75 observed within the PLP10 group (p=0.047). No adverse events were reported. Interventions
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12 76 A and C showed no significant efficacy.
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18 78 **Conclusions** PLP10 treatment significantly reduced the ARR, and the risk of sustained
19 79 disability progression without any adverse or severe side effects.
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23 81 **Trial registration** International Standard Randomized Controlled Trial, number
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92 Introduction

93 Multiple sclerosis (MS) is a complex multifactorial disease that results from interplay
94 between as yet unidentified environmental factors and susceptibility genes.¹⁻³ Together, these
95 factors trigger a cascade of events, involving engagement of the immune system,
96 inflammatory injury of myelin, axons and glia, functional recovery and structural repair,
97 gliosis, and neurodegeneration.⁴ The bio-mechanisms involved are: immune-mediated
98 inflammation, oxidative stress and excitotoxicity.⁵⁻⁹ These mechanisms may all contribute to
99 oligodendrocyte and neuronal damage and even cell death, hence promoting disease
100 progression. The increasing prevalence of MS, the limited efficacy and the side effects of the
101 existing treatments urge the clinical need for the development of new, innovative, more
102 effective, safe, and preventive treatment strategies.

103
104 Research has shown that multiple variables dynamically interact and many different complex
105 interrelated processes are simultaneously orchestrated for MS pathogenesis. The fundamental
106 distinctiveness of **systems medicine (SM)** is not just the recognition that different specific
107 complex factors are important in disease management, but that they need to be incorporated
108 in some meaningful way to treatment selection and delivery.¹⁰ The primary challenge tackled
109 by systems scientific approach is the elucidation of how these multiple variables dynamically
110 interact and how one can apply this understanding to affect the system and achieve a
111 desirable end.¹⁰ The answer might be the simultaneous interference with all involved
112 perturbed mechanisms, by using a cocktail of different specific ingredients, potentially able
113 through synergistic effect to give a long, holistic and effective treatment (Supplementary
114 Information Methods 1).

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3 116 The polyunsaturated fatty acids (PUFA) composition of membrane phospholipids plays a
4
5 117 direct role in immune and non-immune related inflammation. PUFA and antioxidant
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7 118 deficiencies along with decreased cellular antioxidant defense mechanisms have been
8
9 119 reported for MS patients.¹¹ The cause of these PUFA deficiencies is not entirely clear and may
10
11 120 involve metabolic and nutritional alterations.¹¹
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16 122 Increased or uncontrolled inflammation contributes to several different acute and chronic
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18 123 diseases and it is characterized by the production of inflammatory cytokines, arachidonic acid
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20 124 (AA)-derived eicosanoids (prostaglandins (PGs), thromboxanes (TXs), leukotrienes (LTs),
21
22 125 and other oxidized derivatives), other inflammatory agents such as reactive oxygen species
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24 126 (ROS), nitric oxide (NO), and adhesion molecules (Fig 1).¹² During inflammation glutamate
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26 127 homeostasis is altered by activated immune cells releasing increased quantities of glutamate
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28 128 that can result in over activation of glutamate receptors and in return excitotoxic
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30 129 oligodendroglial death.^{7, 13} As such, among others, membrane-related pathology, immune-
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32 130 mediated inflammation, oxidative stress and excitotoxicity provide potentially useful
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34 131 combined targets for intervention in MS.
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40 133 *In vitro and in vivo* studies have demonstrated that dietary EPA, DHA, LA, and GLA can be
41
42 134 implicated and modulate almost all known complex network of events and pathways
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44 135 repertoire in MS pathophysiology. Brain membrane fatty acid composition can be modified
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46 136 with dietary supplementation, but the process has been showed to be age dependent (it takes
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48 137 much longer in adults versus developing brains) as well as possibly dependent on the
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50 138 quantities of the dietary/supplemented PUFAs.¹⁴ Both human and animal studies proved that
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52 139 diets high in DHA and EPA increase the proportion of these PUFA in the membranes of
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54 140 inflammatory cells and reduce the levels of AA.^{12, 15} The anti-inflammatory properties of
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3 141 omega-3 include production of PGs and TXs of the 3-series and LTs of the 5-series (Fig 1).¹⁴
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5 142 ¹⁶ Resolvins and protectins are biosynthesized from omega-3 fatty acids via cyclooxygenase-
6
7 143 2/lipoxygenase (COX-2/LOX) pathways and they promote control of inflammation in neural
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9 144 tissues (Fig 1).¹⁷⁻²¹ T-cell proliferation in acute and chronic inflammation can be reduced by
10
11 145 supplementation with either omega-6 or omega-3 PUFA.²² Furthermore, vitamin E is an
12
13 146 important antioxidant that can interrupt the propagation of free radical chain reactions.²³
14
15 147 Vitamin E (alpha-tocopherol, an isoform of vitamin E) efficiently detoxifies hydroxyl,
16
17 148 perhydroxyl and superoxide free radicals.²⁴ However γ -tocopherol (another isoform of
18
19 149 vitamin E) seems to be more efficiently implicated in trapping NO radicals.²⁵ In addition
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21 150 alpha-tocopherol exerts non-antioxidant properties, including modulation of cell signaling
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23 151 and immune function, regulation of transcription, and induction of apoptosis.²⁶
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29 153 Moreover, omega-3 fatty acids electrophilic derivatives formed by COX-2, in activated
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31 154 macrophages can stimulate the nuclear respiratory factor (Nrf2) inducing the transcription of
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33 155 neuroprotective and antioxidant related genes, and can activate the peroxisome proliferator-
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35 156 activated receptor (PPAR) γ for anti-inflammatory response.²⁷⁻²⁹ In animal studies, EPA and
36
37 157 DHA proved to be endogenous ligands of RXRs, with positive effect on neurogenesis.³⁰
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39 158 Additionally, in 2008 Salvati and coworkers reported evidense of accelerated myelination in
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41 159 DHA- and EPA-treated animals.³² Moreover, DHA and EPA are reported to significantly
42
43 160 decrease the levels of metalloproteinases (MMP) -2, -3, -9, and -13 with a significant role in
44
45 161 the migration of lymphocytes into the CNS by inducing disruption of the blood brain barrier
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47 162 (BBB), an important step in the formation of MS lesions.³³⁻³⁹
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53 164 Based on the above, specific PUFA and antioxidant vitamins fulfill the criterion of biologic
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55 165 plausibility and have the potential to diminish MS symptoms severity and activity, even
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3 166 promoting recovery (remyelination).¹¹ Overall, PLP10 includes multiple ingredients
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5 167 interacting with key interconnected components within functional network modules, each
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7 168 contributing a fraction of the effects of perturbations that cause the disease.⁴⁰
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11
12 170 In our phase II, single-center, randomized, double-blind, placebo-controlled, proof-of-
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14 171 concept clinical trial we intended to evaluate the therapeutic ability of PLP10 and of two
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16 172 other interventions (A and C) consisting of PLP10 constituent partial fractions versus
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18 173 placebo, when used on RRMS patients.
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24 25 176 **Methods**

26 27 177 **Patients**

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30 178 Eligibility criteria were an age of 18 to 65; a diagnosis of RRMS according to the McDonald
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32 179 criteria; a score of 0.0 to 5.5 on the EDSS, a rating that ranges from 0 to 10, with higher
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34 180 scores indicating more severe disability; MRI showing lesions consistent with MS; and at
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36 181 least one documented clinical relapse either receiving or not disease modifying treatment
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38 182 (DMT) within the 24 months period before beginning (enrollment) of the study. Patients were
39
40 183 excluded because of a recent (<30 days) relapse, prior immunosuppressant or monoclonal
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42 184 antibodies therapy, pregnancy or nursing, other severe disease compromising organ function,
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44 185 progressive MS, history of recent drug or alcohol abuse, use of any additional food
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46 186 supplement, vitamin, or any form of PUFA, history of severe allergic or anaphylactic
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48 187 reactions or known specific nutritional hypersensitivity. No monitor or limitations on
49
50 188 patients' daily diet habits were included in the study design since the quantities of the
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52 189 ingredients within the formulas daily-dosage could not be significantly affected or spoiled by
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3 190 any confounding factors within any known global daily food diet (see procedures, treatment
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5 191 regimen and end-points).
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9 193 The study was conducted in accordance with the standards of the International Conference of
10
11 194 Harmonization Guidelines for Good Clinical Practice. The protocol was developed by the
12
13 195 investigators and it was approved by the Cyprus National Bioethics Committee and was
14
15 196 overseen by an independent safety-monitoring committee evaluating the safety and over-all
16
17 197 benefit-risk profiles. The adherence of care providers with the protocol was assessed by an
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19 198 external committee assigned by the funder of the project through reviews of case report
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21 199 forms. All patients gave written informed consent at the time of enrolment.
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25 200
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27 201 **Randomization and masking**

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29 202 Patients were randomly assigned to four intervention groups in a 1:1:1:1 ratio stratified by
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31 203 gender (women to men, 3:1). Randomization was facilitated by a lottery-type pool of
32
33 204 numbered balls. Patients were randomly assigned to treatment in blocks of four by flipping a
34
35 205 coin as follows: for the first two drawn balls heads stratified them to the groups A/B and tails
36
37 206 stratified them to the groups C/D. The other two balls were stratified accordingly. A second
38
39 207 toss of the coin assigned the two patients to group A (head)/B (tail) or group C (head)/D
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41 208 (tail). The randomization scheme was generated, performed and securely stored by Helix
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43 209 Incubator Organization of Nicosia University (HIONU).
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49 211 The interventions had identical appearance and smell in dark bottles (15 daily-dose
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51 212 portions/bottle) under nitrogen bed and labeled by HIONU with code numbers, unidentifiable
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53 213 for both patients and investigators. Study data were collected by the investigators and saved
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55 214 by the HIONU that also held the blinded codes of the study. All study personnel involved in
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3 215 the conduct of the study were blinded throughout the study. Treating/examining physician,
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5 216 other investigators, pharmacist, neuroradiologist and patients were masked to treatment
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7 217 allocation.
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11 219 **Procedures and end points**

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14 220 The specific omega-3 (re-esterified glycerides) and omega-6 (glycerides) raw materials were
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16 221 purchased according to the required interventions' PUFA-fraction specification (molecular
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18 222 structure, quantity/ratio and quality) with vitamin E (alpha-tocopherol) used as antioxidant
19
20 223 stabilizer by the supplier. The vitamins and masking aroma were purchased separately. The
21
22 224 mixing of fractions to the final required intervention-composition specification was always
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24 225 performed by the same team of scientists under the supervision of the involved medical
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26 226 biochemist and lipidology specialist, under appropriate conditions every six months.
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28 227 Interventions were stored refrigerated in dark until use. See **Table 1** and Supplementary
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30 228 Information Methods 1 and 2 for intervention specification detailed description and
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32 229 study/intervention rational.
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38 231 Participants were randomly assigned to receive a daily dose of a mixture of EPA (1,650mg) /
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40 232 DHA (4,650mg) / GLA (2,000mg) / LA (3,850mg) / total other omega-3 (600mg) / total
41
42 233 MUFA (1,714mg) + total SFA (18:0 160mg, 16:0 650mg) / vitamin A (0.6mg) / vitamin E
43
44 234 (22mg) (intervention A, group A); or composed mixture of pure γ -tocopherol (760mg)
45
46 235 dispersed in pure virgin olive oil (16,137 mg) as delivery vehicle (intervention C, group C);
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48 236 or a mixture of intervention formula A with intervention C without the pure virgin olive oil
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50 237 (intervention B, named PLP10, group B); or placebo composed of pure virgin olive oil
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52 238 (16,930mg) (intervention D, group D) (Table 1). Citrus-aroma was used as masking agent of
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54 239 the taste and odor and added in each one of the intervention for a total of 19.5ml dosage of
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3 240 solution per day. The institution's pharmacist was responsible for the appropriate storage and
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5 241 handling of the interventions to the individual participants. The interventions were taken
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7 242 orally once daily 30 minutes before dinner by a dosage calibrated cup for 30 months. The
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9 243 ingredients, ratio and dose have been selected based on their biophysical interrelation to the
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11 244 total known multiple MS causing factors, their biochemical importance and the role expected
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13 245 to play in the normalisation and treatment of the involved complex network of events in the
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15 246 disease pathophysiology. Moreover, the high intake dosage was used to overcome any
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17 247 abnormal dietary accumulation of related agents as a result of patients' food intake habits,
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19 248 irrespective of geographical origin, in relation to the daily consumption ratio of the total fatty
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21 249 acid intake; in order to end-up with omega-3 to omega-6 PUFA indicated physiological body
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23 250 ratio composition of 1:1 wt/wt.
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30 252 The period beginning from July 1st 2007 (enrolment) until December 31st 2007 (entry
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32 253 baseline) was used for normalization period. This six-month normalization period would
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34 254 allow the interventions' agents to exert their beneficial effect (for the
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36 255 incorporation/normalization of cell membranes by oral PUFA, since they need four to six
37
38 256 months to exert pivotal action on immune and neural cells, correction of antioxidant
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40 257 deficiency and body PUFA, and optimal normalization of EPA and DHA levels/ratio).⁴¹⁻⁴³
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42
43 258 The study was completed on December 31st 2009 and the recording of relapses continued
44
45 259 until December 31st 2010. More clearly the study included the "normalization period" (July
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47 260 1st 2007 to Dec 31st 2007), the "on treatment" period (Jan 1st 2008 to Dec 31st 2009) and the
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49 261 12-month "extended period" (Jan 1st 2010– Dec 31st 2010).
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54 263 Depending on their clinical status and in accordance with the ethical issues governing clinical
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56 264 trials participants continued receiving the indicative regular available treatments, according to
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3 265 international guidelines with persistent evaluation of any side-effects and adverse events.

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5 266 The study was designed to end 30 months after enrolment and clinical assessments were

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7 267 scheduled at entry baseline, 3, 9, 15, 21 and 24 months on-treatment. Patients were also

8
9 268 clinically examined by the treating neurologist within 48 hours after the onset of new or

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11 269 recurrent neurologic symptoms.

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16 271 The primary end point was the ARR at two years. A relapse was defined as new or recurrent

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18 272 neurologic symptoms not associated with fever or infection that lasted for at least 24 hours

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20 273 and accompanied by new neurologic signs. Relapses were treated with methyl-prednisolone

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22 274 at a dose of 1g intravenous per day, for three days followed by prednisone orally at a dose of

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24 275 1mg/kg of weight per day on a tapering scheme for three weeks. The secondary end point at

25
26 276 two years was the time to confirmed disability progression, defined as an increase of 1.0 or

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28 277 more on EDSS, confirmed after six months (progression could not be confirmed during a

29
30 278 relapse). The final EDSS score was confirmed six months after the end of the study. A post-

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32 279 hoc analysis was performed assessing the proportion of patients free from new or enlarging

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34 280 T2 lesions on brain MRI scans at the end of the study for the per-protocol participants of the

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36 281 group receiving the highest effective intervention versus placebo. Comparison was made only

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38 282 versus the available archival MRI scans up to three months before the enrolment date. MRI

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40 283 scans were performed and blindly analyzed at an MRI evaluation centre. The patients

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42 284 continued to be followed for additional 12 months after completion of the trial and relapses

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44 285 were recorded. Finally, patients were strongly encouraged to remain in the study for follow-

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46 286 up assessments even if they had discontinued the study drug.

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53 288 Blood samples were collected from all randomized patients at the time of enrolment, at every

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55 289 scheduled clinical assessment and during relapses. To check individual compliance with

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3 290 intake, the fatty acids composition of patients' red blood cells' membranes was determined,
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5 291 by gas chromatography, according to a standard protocol. The fatty acid analyses were
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7 292 performed after study termination and thus did not influence the blinding.
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11 294 The involved neurologist was experienced with more than 20 years in practice and trained to
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13 295 standardise EDSS scoring procedures, examined patients, made all medical decisions,
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15 296 determined the EDSS score and reviewed adverse or side-effects. The medical biochemist,
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17 297 specialist on lipidology and immunology and the registered clinical dietitian, members of the
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19 298 investigator team were experienced with more than 25 years in practice. Patients were able to
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21 299 contact the neurologist at any time if there was any adverse event, side-effect or allergic
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23 300 reaction. The study drug was not suspected to have any clinical or laboratory adverse effects
24
25 301 different from placebo that could disturb the double-blind nature of the trial. Therefore, the
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27 302 same study-neurologist functioned as both the treating and evaluating physician.
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34 304 Safety measures were assessed from the time of enrollment until 12 months following study
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36 305 completion. Haematological and biochemical tests were performed at enrolment and at every
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38 306 12 months, including full blood count, renal and liver function tests, proteins, cholesterol,
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40 307 triglycerides, glucose and electrolytes.
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45 309 The whole procedure followed the clinical trial guidelines as required by the USA Food and
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47 310 Drug Administration, European Medicines Agency, and the Committee for Medicinal
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49 311 Products for Human Use.⁴⁴
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54 313 **Statistical analysis**
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3 314 Power calculations could not be done before the study because of the lack of information
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5 315 from previous studies on potential effect sizes. In 2005 the prevalence of MS in Cyprus
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7 316 (600,000 population) was 120/100.000. Based on the aforementioned MS patients' numbers
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9
10 317 of our country and the centre of reference, the CING, we were able to enrol the 20% of the
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12 318 total RRMS patients eligible for treatment in the trial. Sample size was strictly based on this
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14 319 subjects' availability parameter and the novelty of the assessed intervention.
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18 321 Baseline characteristics were compared across all intervention groups by ANOVA or
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20 322 Kruskal-Wallis rank test for continuous variables and by mean Fisher's exact test for
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22 323 categorical variables, as appropriate.
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27 325 For the primary outcome, the ARR was analysed in a pair-wise fashion for the active
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29 326 interventions compared to placebo using negative binomial regression models adjusted for
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31 327 number of relapses within two years before baseline, EDSS score at baseline and DMT. The
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33 328 relapse rate was calculated as the total number of relapses divided by the total number of
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35 329 patient-years followed for each treatment group. ARR differences were also calculated
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37 330 among all comparable parameters and reported as percent difference.
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42 332 For the secondary end-point outcome, the time to disability progression, Kaplan–Meier
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44 333 curves were constructed. Progression to disability and time thereof was compared in a pair-
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46 334 wise fashion for the active interventions versus placebo by the log-rank test in the main
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48 335 analysis and by Cox proportional-hazards models with adjustment for baseline EDSS score,
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50 336 age and DMT in the supportive analysis. Each test was performed with a significance level of
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52 337 0.05. Multivariate models considered all variables with $P < 0.1$ on univariate models. There
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54 338 was no overt violation of the proportionality assumption.
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5 340 Both, per-protocol and intention to treat (ITT) analyses were performed, for different sets of
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7 341 research questions to be answered, and both are reported. Missing data of the five lost to
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9 342 follow patients were imputed by use of the last-observation-carried-forward (LOCF)
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11 343 approach. Due to the proof-of-concept design of the study, the considerable non-adherence
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13 344 rate (49%) and the interpretation issues caused thereof regarding the ITT analysis, the per-
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15 345 protocol analysis considered being more informative and appropriate method approach to
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17 346 answer the research addressed questions of efficacy of the interventions when subjects were
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19 347 continuously following the protocol. All statistical analyses were well defined a priori. All
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21 348 analyses were performed with STATA SE 10.0 (College Station, TX, USA). P-values are
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23 349 two-tailed.
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351 **Role of the funding source**

352 The funders had no role in study design, data collection and analysis, decision to publish, or
353 preparation of the manuscript. All members of the writing group had full access to all study
354 data and contributed to its interpretation and prepared, reviewed, and approved the
355 manuscript for submission. All authors had final responsibility for the decision to submit the
356 paper for publication.
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358 **Results**

359 **Study population**

360 From July 2007 through December 2010 (including the 12-month extended period), a total of
361 80 MS patients were randomly assigned to a study group at the CING (tertiary neurological
362 center).
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3 364 Among the 80 patients, 20 patients were randomly assigned to each of the three groups to
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5 365 receive the interventions and 20 to receive placebo (Fig 2). Baseline characteristics of both
6
7 366 the ITT and the per-protocol populations were similar across groups (Table 2A and 2B).
8
9 367 Total drop-out patients completed follow-up until study completion and were included in the
10
11 368 ITT analyses (Table 4). Five patients were totally lost to follow before their first scheduled
12
13 369 visit and two patients dropped-out before their first scheduled visit progressed to secondary
14
15 370 progressive MS. Fifteen patients dropped-out without successfully completing the
16
17 371 “normalization” period including five pregnancies. Another 17 patients dropped-out early
18
19 372 after entry baseline. Seven patients that dropped out were given monoclonal antibody
20
21 373 treatment (natalizumab). Overall, a total of 41 (51%) patients completed the 42-month study
22
23 374 (July 2007 through December 31st 2010, including the 12-month extended period) where one
24
25 375 patient from group A and two from the placebo group transferred on natalizumab, and 39
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27 376 (49%) patients either withdrew (drop-out) or lost to follow. Reasons for study-interventions
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29 377 discontinuation are listed in Figure 2.
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36 379 **Efficacy**

38 380 **Relapses**

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40 381 As a proof-of-concept trial we primarily needed to answer whether the interventions were
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42 382 effective for those MS patients who adhere to the assigned treatment, the per-protocol
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44 383 analysis.⁴⁵ For the sake of methodological comprehensiveness we also present the ITT
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46 384 analysis as a secondary analysis, to answer a different question, complementary to our core
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48 385 hypothesis; like what happened to MS patients who were placed on the interventions (the
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50 386 effect of assignment).⁴⁵ Otherwise, as a result of a high drop-out rate, an ITT analysis will not
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52 387 likely be able to show the superiority of an intervention even if it is effective.⁴⁵ In any
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54 388 instance, the proper approach of evaluating a study data is to understand what question
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3 389 prompted the research and assure that the analysis is appropriate for providing the answer
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5 390 whatever it is called. Both analyses can be performed for a study, using the results from the
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7 391 different analyses to answer different research questions.⁴⁵ These interventions are original,
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9 392 composed by a different treatment rational, the SM, never tested before and the important
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11 393 main concern was to evaluate their efficacy and safety based on the per-protocol treated MS
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13 394 patients, without any peripheral noise. The question that had to be answered was: “what
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15 395 happens to the patients that are placed and stick on the specific treatment”.

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21 397 Regarding the per-protocol analysis, during the first year of treatment, the ARR was 0.80,
22
23 398 0.40, 0.78 and 0.83 for the four intervention groups respectively. During the second year, the
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25 399 ARR was 0.90, 0.40, 0.67 and 1.25 for the four intervention groups respectively. Overall, for
26
27 400 the two-year primary end point, 8 relapses were recorded for the 10 patients in PLP10 group
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29 401 (0.40 ARR) versus 25 relapses for the 12 patients in placebo (1.04 ARR), a 64% adjusted
30
31 402 relative rate reduction (RRR) for the PLP10 group (RRR 0.36, 95% confidence interval (CI)
32
33 403 0.15 to 0.87, p=0.024) (Tables 3A, 5 and Fig 3A and 3C). Excluding patients on monoclonal
34
35 404 antibody (natalizumab) treatment, the observed adjusted RRR became stronger (72%) over
36
37 405 the two years (RRR 0.28, 95% CI 0.10 to 0.79, p=0.016, Tables 3B and 5). Pair-wise
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39 406 comparisons for the other two groups against placebo did not yield statistically significant
40
41 407 results (Tables 3A, 3B). The proportion of patients with ≤ 1 relapse for the two years on-study
42
43 408 was higher in the PLP10 group than in the placebo group (90% versus 42%, p=0.030, Table
44
45 409 5). Seeking to investigate further the observed difference, we compared the relapse rate
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47 410 during the 24 months before entry to the study to the 24 months on-treatment for each
48
49 411 intervention group. We observed a statistically significant relative reduction in the ARR
50
51 412 (70%) only in the PLP10 group (RRR 0.30; 95% CI 0.14 to 0.65, p=0.003, Table 3A);
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53 413 within-group comparisons for the three other groups ARR reduction was not significant and
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3 414 remained not significant when natalizumab treated patients were further excluded from the
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5 415 analysis. The effect of PLP10 through time at different time-windows versus placebo for all-
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7 416 time on-study patients is shown in Figures 3A to 3D. The ARR analysis, within time-
8
9 417 windows, was not an assigned endpoint, but it could help in the process of evaluating parallel
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11 418 information as the time needed for a specific treatment intervention activity to be evident, as
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13 419 well as the efficacy profile through time. PLP10 reached maximum effect within a year on-
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15 420 treatment (counting from the entry baseline) and remained stable at an ARR of 0.4,
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17 421 displaying a steadily reduced ARR with long free-relapse time-windows. These group B
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19 422 characteristics are considered important parameters of a successful MS treatment where the
20
21 423 rule than the exception is the heterogeneity among patients' disease evolution. Specifically,
22
23 424 Figure 3D demonstrates the dispersion of relapses throughout the 2-year period of all-time
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25 425 on-study (excluding patients on natalizumab) of PLP10 (n=10) versus placebo (n=10).
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27 426 Placebo, in line with the existing knowledge of how relapse history works in relation to future
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29 427 relapses on MS patients (contagion phenomenon) indicates the expected linearly increased
30
31 428 trend of the relapse incidence.⁴⁶ The same phenomenon was true for the groups A and C.
32
33 429 Finally, during the 12 month post-study extended period (January 1st 2010 to December 31st
34
35 430 2010) all-time on-study patients that received PLP10, showed persistent benefit in the ARR
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37 431 compared to placebo (six relapses for the 10 subjects within PLP10, 0.6 ARR versus 1.9 for
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39 432 the 12 subjects within placebo group, 1.58 ARR) indicating a statistically significant 62%
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41 433 adjusted relative rate reduction in the ARR for the PLP10 group (RRR 0.38, 95% CI 0.12 to
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43 434 0.99, p=0.046).
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51 436 Regarding the ITT analysis, within PLP10 group, none of the nine drop-out patients changed
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53 437 to natalizumab and two out of nine discontinued because of pregnancy, whereas two out of
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55 438 seven drop-out patients from the placebo group changed to natalizumab (a total of four
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3 439 patients within the placebo arm population were on natalizumab, including the two patients
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5 440 that transferred while all-time on-study versus none within PLP10 group (Supplementary
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7 441 Information Fig 1). As reported, natalizumab reduces ARR by 68%, decreases the possibility
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9 442 of disability progression by 43% with 57% of patients free of new or enlarging T2 lesions on
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11 443 MRI scans compared to 15% on placebo.⁴⁷ The relapses of the drop-out patients are reported
12
13 444 in Table 4A. As expected no statistically significant differences in the ARR were calculated
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15 445 for the comparison of any group versus placebo for the 24 months on-treatment, with a 0.75
16
17 446 ARR within PLP10 (30 relapses) and 1.03 ARR within placebo (41 relapses) group, a 27%
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19 447 ARR reduction (Table 4B). Interestingly, despite the high non-adherence rate, there was a
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21 448 statistically significant difference for the comparison of the ARR in the 24 months before
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23 449 entry baseline to the 24 months on-treatment for the PLP10 group (RRR 0.45, 95% CI 0.26 to
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25 450 0.78, $p=0.005$).

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31 452 **Disability progression**

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33 453 Regarding the per-protocol analysis, at two years, the time to disability progression, with
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35 454 confirmation after six months (secondary end-point) was significantly longer only with
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37 455 PLP10. The cumulative probability of disability progression was 10% in the PLP10 group
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39 456 and 58% in the placebo group ($p=0.019$) (Supplementary Information Fig 2). After excluding
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41 457 patients on natalizumab, there was an increased statistically significant difference between
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43 458 the PLP10 and the placebo group for the same analysis ($p=0.006$) (Fig 4A). At two years, the
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45 459 cumulative probability of progression was 10% in the PLP10 group and 70% in the placebo
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47 460 group, which represents a decrease of 60 percentage points or a relative 86% decrease in the
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49 461 risk of sustained progression of disability within PLP10 group (adjusted hazard ratio, 0.11;
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51 462 95% CI 0.01 to 0.97, $p=0.047$). One versus seven out of ten patients progressed to confirmed
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53 463 disability in the PLP10 and the placebo groups respectively when patients on natalizumab
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3 464 were excluded. No statistically significant difference was observed for any comparison of the
4
5 465 other two groups compared to placebo (Fig 4A and Supplementary Information Fig 2).
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10 467 Regarding the ITT analysis, at two years, the cumulative probability of progression was 10%
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12 468 in the PLP10 group and 35% in the placebo group ($p=0.052$, a trend for an effect), which
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14 469 represents a decrease of 25 percentage points or a relative 71% decrease of the PLP10 for the
15
16 470 risk of sustained progression of disability (adjusted hazard ratio 0.22, 95% CI 0.04 to 1.07,
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18 471 $p=0.06$) (Fig 4B). Two versus seven out of the total randomized patients progressed to
19
20 472 confirmed disability in the PLP10 and the placebo groups respectively. No significant
21
22 473 differences were observed for groups A or C against placebo (Fig 4B). The mean change in
23
24 474 Expanded Disability Status Scale (EDSS) score as a function of visit number is shown in
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26 475 Figure 5.
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31 477 **MRI**

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34 478 Over two years, the MRI results support the overall conclusion from the study that PLP10 has
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36 479 a positive effect on disease activity since only 29% from the PLP10 group as opposed to 67%
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38 480 from the placebo group developed new or enlarging T2 lesions (57% relative risk reduction).
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40 481 Excluding patients on natalizumab there is an increased relative risk reduction (64%) between
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42 482 PLP10 as opposed to placebo with 29% of patients on PLP10 and 80% on placebo with
43
44 483 development of new or enlarging T2 lesions (Table 5).
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48 485 **Safety**

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51 486 Over the course of the 30 month study no significant adverse events were reported from any
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53 487 group. According to a questioner procedure the only aetiology for drop-outs was the
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55 488 palatability and smell of the formula preparations. Nausea was reported by two patients. No
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3 489 abnormal values observed on any of the biochemical and haematological blood tests. No
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5 490 allergic reactions reported.
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9 10 492 **Discussion**

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12 493 In this proof-of-concept clinical trial assessing the safety and efficacy of three cocktail
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14 494 intervention formulas in RRMS, we observed a significant benefit for the novel PLP10
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16 495 intervention compared to placebo for both the ARR and the progression to disability. Our
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18 496 results include analyses pertaining to a total of 42 months study collected data, including the
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20 497 12-month, free of intervention treatment, extension period. We focused on the per-protocol
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22 498 data analysis since it is the appropriate method to best provide the answer to the proof-of-
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24 499 concept trial-addressed question. The high drop-out rate was solely the result of formulas
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26 500 palatability, a common phenomenon in trials using oily interventions **where a lot of patients**
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28 501 **tend to drop-out soon after first dosage**. We thus present our main per-protocol analysis, as
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30 502 well as a subgroup analysis excluding patients on natalizumab. We have found a statistically
31
32 503 significant reduction in the ARR and the disability progression comparing not only patients
33
34 504 on PLP10 versus placebo but also comparing the ARR of the PLP10 patients in the 24-month
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36 505 period prior to the study to the ARR of the 24 months on-study; the observed differences
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38 506 became larger when patients that received natalizumab (the most potent disease modifier)
39
40 507 were excluded. The ARR decreased within a year on PLP10 and significantly remained stable
41
42 508 until study completion. Statistically significant difference of ARR between patients on PLP10
43
44 509 versus placebo continued for the additional 12 month extended period (persistent effect)
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46 510 without significant difference on DMT. These clinical findings are supported by the results
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48 511 regarding the MRI analysis where the proportion of patients free from new or enlarging brain
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50 512 T2 lesions was also higher in PLP10 group versus placebo. The persistent effect within the
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52 513 extended period it is considered of major importance and supportive of the results since it is
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3 514 in agreement with the very long washouts, reported necessary, for omega-3 fatty acids and
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5 515 especially DHA to return towards pretreatment values within the fatty acids of plasma,
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7 516 platelets, monocytes and red blood cells.⁴² This study also provides important 30-month,
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10 517 placebo-controlled information about the safety of PLP10, A and C interventions, where no
11
12 518 any adverse or severe side effects have been reported.
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16 520 As medications used to treat MS become increasingly highly specific and potent, attention to
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18 521 safety is paramount. Current available treatments are products of reductionism, partially
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20 522 effective, associated with severe side effects without (re)myelinating or neuroprotective
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22 523 abilities. Interferons and glatiramer acetate, the most widely used first-line MS drugs
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24 524 available today, are associated with the least severe side-effects among MS therapies but they
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26 525 are reported with only 29-33% ARR reduction and with no significant effects on the
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28 526 progression of disability. Natalizumab as previously discussed and Fingolimod with 54%
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30 527 ARR reduction (without significant benefit on the progression of disability) are second-line
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32 528 drugs associated with severe side-effects.^{47, 48}
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38 530 No existing MS treatment has ever been designed as a result of SM concept approach or with
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40 531 a potential to effectively stimulate intrinsic remyelinating and neuroprotecting mechanisms or
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42 532 exert such an action. Now we propose that a holistic SM model approach has to be applied by
43
44 533 synchronized action on all involved perturbed mechanisms. PLP10 has innovative
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46 534 characteristics like no any other intervention or medication tried before for MS treatment,
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48 535 with unique efficacy abilities through different mechanisms of action, probably by the
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50 536 synergistic effect of its constituent ingredients. PLP10 has all the characteristics of a medical
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52 537 food with the action to feed a normal metabolic process by supplying nutritional structural
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54 538 membrane precursors, building blocks, and vitamins from dietary sources that enhance
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3 539 remyelination and neuroprotection and simultaneously promote normalization of all cellular
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5 540 membranes lipid content. The intention is to normalize the specific nutritional requirements
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7 541 of the MS patients.
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11 543 Different factors and molecular entities appear to be part of the possible aetiology for MS
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13 544 with specific PUFA and antioxidants found to be key substances related to all known
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15 545 pathogenic and recovery mechanisms. But, it is well established that MS patients are
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17 546 characterized with significant deficiencies in antioxidant efficiency as well as in PUFA levels
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19 547 in blood and cellular membranes.^{11, 49-51}
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25 549 According to one hypothesis, the change in the ratio of omega-3 to omega-6, due to
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27 550 increasing consumption of omega-6 PUFA, meaning high accumulation of AA, in the
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29 551 Western diet, may be one of the major factors responsible for the increasing incidence of
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31 552 inflammatory diseases relative to populations. In the Western diet, the ratio of omega-3 to
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33 553 omega-6 is about 1:20–30; in populations that consume fish-based diets, the ratio is about
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35 554 1:1–2.^{52, 53} The intervention daily dose was aiming and believed to be high enough to
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37 555 restore/amplify body efficient antioxidant activity and ensure cellular membranes lipid profile
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39 556 normalization (PUFA content) and simultaneously potentiate involvement of the ingredients
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41 557 in the anti-inflammatory and recovery mechanisms. Diet fatty acid molecules need about six
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43 558 months period to exert their beneficial effect and this essential parameter was for the first
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45 559 time under consideration in our study design (normalization period).⁴² This chronotherapy
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47 560 parameter it is of major importance in line with the SM treatment philosophy and if it is not
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49 561 included in the trial design the possibility of misleading result evaluation greatly increases. In
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51 562 fact, considering that omega-3 supplementation can release and replace excess AA within the
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3 563 cellular membranes, we can speculate that an increased inflammatory activity can possibly
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5 564 result during the first six months of supplementation (during normalization period).
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10 566 The maintenance of myelin requires continued turnover of its components throughout life.^{54,55}

11 567 In addition to the EPA, DHA, LA, and GLA, PLP10 was containing limited quantities of

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13 568 other structural PUFA, specific MUFA (mostly oleic acid) and SFA (palmitic and stearic

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16 569 acid), specifically to provide a direct source for neuronal cell membranes rehabilitation and

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18 570 for (re)myelination and neuroprotection since they are all major components, precursors and

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21 571 building blocks of any new physiological myelin and cellular membranes in general.

22 572 Assembly of the correct molecules into myelin membrane may be especially critical during

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25 573 active synthesis. Possibly, if critical constituents aren't available or are metabolically

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27 574 blocked, amyelination, dysmyelination or demyelination may ensue.⁵⁶
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32 576 The well known and established safety of the ingredients used and the protocol guidelines

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34 577 were supportive reasons for us to proceed with the clinical study even though with limitation

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36 578 on the pre-estimation of required trial sample size as it was discussed in method section. The

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38 579 adherence of the subjects is another issue but the duration of the study (42 months) is adding

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40 580 power to the results;⁴⁴ having the research questions been consciously and carefully

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43 581 approached and answered. Furthermore, the statistical methodologies used along with the

44
45 582 appropriate adjustments, broadly accepted for MS clinical trials, power the findings, results,

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47 583 and significance. The baseline characteristics of the treatment arms could possibly be

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49 584 considered indicative of four very active groups of patients but that was the result of the

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51 585 limited number of RRMS population eligible for the study within Cyprus. On the other hand

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54 586 the balanced baseline characteristics without statistical differences, the statistical adjustments

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56 587 (for all important baseline parameters i.e. relapses, EDSS, age and DMT) and the
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3 588 randomization within four different groups are the safety valves against data
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5 589 misinterpretation. Yet, in small randomized control trials with a high drop-out rate, the per-
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7 590 protocol analysis could be affected by the characteristics of the patients dropping out. In
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9 591 order to safeguard our findings in the best possible way under the circumstances, we
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11 592 proceeded to adjusting for confounders. Moreover, we cannot discard our finding as a false
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13 593 positive, given that this is a randomized, double-blind, placebo-controlled clinical trial and,
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15 594 despite its small sample size, represents a piece of evidence that only a larger randomized
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17 595 controlled trial can replicate or refute. It is possible to question why DMTs efficacy cannot
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19 596 be emerged out of the data analysis, of the four treatment arms, and in accordance to their
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21 597 published values. We believe that the limited efficacy of the DMTs, the sample size and the
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23 598 statistical adjustments were strong limiting determining factors for such an indication to be
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25 599 countable. An additional argument is that the efficacy reported for the analysis of pre-
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27 600 treatment (24 months before entry baseline) versus on-trial ARR could be considered as
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29 601 potentially biased due to differences of how relapses were defined during the course of a
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31 602 study compared to pre-treatment period; or due to regression to the mean or placebo effect.
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33 603 This analysis was performed as an additional exploratory analysis that we were able to do due
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35 604 to the availability of data. The relapses of the two pre-treatment years were drawn out of the
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37 605 patients' archival records by the same treating neurologist involved in the study (MP), and
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39 606 according to the patients' hospitalization date for receiving intravenous methyl-prednisolone.
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41 607 This analysis was not used as a primary or a secondary end-point under investigation
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43 608 although it is usually reported by many clinical studies. As a matter of fact many early phase
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45 609 trials are based only on such an analysis (before versus after treatment results). In almost all
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47 610 MS trials the number of relapses within the two years before baseline is a factor under
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49 611 adjustment for the statistical analyses.⁴⁸ The inclusion of the post-hoc MRI analysis is another
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51 612 limiting factor that needs attention since it was used as an additional aside exploratory
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3 613 approach (due to study budget limitations it was not possible to be used as a formal
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5 614 endpoint); but the MRI evaluation was blinded and can be considered as representative of the
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7 615 randomized subjects within the treatment arms. As far as the regression to the mean and the
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9 616 placebo effect concerns we believe that the 6-month normalization period is an accountable
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11 617 and valuable eliminating factor of the possible effect; as well as the presence of four groups,
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13 618 where only the PLP10 treatment arm is associated with statistically significant efficacy versus
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16 619 placebo. It is a placebo-controlled study after all.
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21 621 Our observations are consistent with the idea that simultaneous availability of specific PUFA
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23 622 along with other major membrane and myelin building blocks in combination with specific
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25 623 antioxidants, within optimum quantity, quality, ratio and structural form can possibly result to
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27 624 a more appropriate holistic therapy reducing MS disease activity. This is probably succeeded
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29 625 through synergistic and/or simultaneous effect on the interactions and dynamics of the most
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31 626 probable environmental and biological disease causing factors that induce complex biological
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33 627 network of events for disease pathogenesis and evolution; as well as on the protective and
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35 628 reparative mechanisms. We can additionally speculate that the nature of the intervention
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37 629 formula cannot be prohibitive for its use as preventive regimen and does not preclude
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39 630 probable positive efficacy on the other types of MS, but has to be further investigated. A
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41 631 larger size multicenter clinical trial will better establish PLP10 place in the armamentarium of
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43 632 treatments for MS.
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634 It is commonly accepted that nutrition is one of the possible environmental factors involved
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636 in the pathogenesis of MS, but its role as complementary MS treatment is unclear and largely
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638 disregarded.⁵⁷ It is well known that the majority of the patients suffering from MS they do
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640 use dietary supplements for a variable length of time and they prefer supplement type of

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3 638 “help” over conventional drugs.⁵⁸ Dietary antioxidants and fatty acids may influence the
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5 639 disease process in MS by reducing immune-mediated inflammation, oxidative stress and
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7 640 excitotoxic damage.¹¹ Present data reveal that healthy dietary molecules have a pleiotropic
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9 641 role and are able to change cell metabolism from anabolism to catabolism and down-regulate
10
11 642 inflammation by interacting with enzymes, nuclear receptors and transcriptional factors.⁵⁷
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13
14 643 The present study, for the first time provides strong link evidence between dietary, metabolic,
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16 644 immunological, and neurobiological aspects of MS after three quarters of a century of
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18 645 unsuccessful scientific efforts. This might probably be the beginning of opening new
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21 646 horizons and new avenues in the approach of MS prevention and treatment, and possibly of
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23 647 other multifactorial chronic diseases, including neurodegenerative and autoimmune as well.
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1.			
Treatment Arms			
A	B (PLP10)	C	Placebo
Intervention: EPA (1,650mg) / DHA (4,650mg) / GLA (2,000mg) / LA (3,850mg) / total other omega-3 (600mg)* / total MUFA** (1,714mg) + total SFA (18:0 160mg, 16:0 650mg) / vitamin A (0.6mg) / vitamin E (22mg)	Intervention: EPA (1,650mg) / DHA (4,650mg) / GLA (2,000mg) / LA (3,850mg) / total other omega-3 (600mg)* / total MUFA** (1,714mg) + total SFA (18:0 160mg, 16:0 650mg) / vitamin A (0.6mg) / vitamin E (22mg) + pure γ -tocopherol (760mg)	Intervention: pure γ -tocopherol (760mg) dispersed in pure virgin olive oil (16,137 mg) as delivery vehicle	Intervention: Olive oil (pure virgin)
* Other omega-3: C18:3n-3 37mg, C18:4n-3 73mg, C20:4n-3 98mg, C22:5n-3 392mg			
** MUFA: 18:1 1300mg, 20:1 250mg, 22:1 82mg, 24:1 82mg			
EPAX1050, EPAX AS, Aalesund, Norway; was used as the source for the omega-3 PUFA, as re-esterified glycerides from fish body oils; Borage seed oil (organic, cold pressed) "Borago officinalis" Goerlich Pharma International GmbH, Edling, Germany, was used as the source for the omega-6 PUFA, MUFA and SFA, as triglycerides. The pure γ -tocopherol was purchased from Tama Biochemical Co. Ltd., Shinjuku-ku Tokyo, Japan; vitamin A as beta-carotene from HealthAid Ltd., Middlesex, United Kingdom and the Citrus aroma from Givaudan Schwaiz AG, Dubendorf, Switzerland.			
Table 1. Intervention ingredients per treatment arm. Citrus-aroma was used as masking agent of the taste and odor and added in each one of the intervention for a total of 19.5ml dosage of solution per day.			

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2A.					
Characteristics	Group A (n=20)	Group B† (n=20)	Group C (n=20)	Placebo (n=20)	P- value
Sex					
Female - no. (%)	15 (75)	15 (75)	15 (75)	15 (75)	1.000
Age (yr)					
Mean ± Standard Deviation (SD)	38.0±11.9	36.9±8.4	37.7±8.7	38.1±10.9	0.982
Median (Range)	38.0 (22–65)	37.0 (25–61)	36.5 (24–54)	36.0 (21–58)	
Treatment history					
Patients on DMT- no. (%)	11 (55)	9 (45)	12 (60)	10 (50)	0.875
Pre-treatment disease duration (yr)					
Mean ± SD	9.0±7.6	8.6±4.8	8.6±5.3	7.7±5.7	0.909
Median (Range)	7.5 (2–37)	8.0 (2–20)	8.0 (3–24)	6.5 (2–25)	
Pre-treatment relapses‡					
Mean ± SD	2.33±1.68	2.41±1.73	2.31±1.66	2.10±1.32	0.946
Median (Range)	2.0 (1–6)	2.0 (1–7)	2.0 (1–6)	2.0 (1–4)	
ARR	1.17	1.21	1.16	1.05	0.946
Patients -% with ≤1 relapse	40	45	40	35	
Baseline EDSS score‡					
Mean ± SD	2.52±1.23	2.15±1.05	2.42±1.21	2.39± 0.93	0.775
Median (Range)	2.5 (1.0–5.5)	2.0 (1.0–4.0)	2.5 (0.0–5.0)	2.5 (1.0–4.0)	
2B.					
Characteristics	Group A (n=10)	Group B† (n=10)	Group C (n=9)	Placebo (n=12)	P- value
Sex					
Female - no. (%)	5 (50)	7 (70)	6 (66.6)	10 (83.3)	0.419
Age (yr)					
Mean ± SD	36.6±13.5	34.80±5.4	40.9±8.1	39.8±13.2	0.572
Median (Range)	34.5 (22–65)	34.5 (26–43)	40.0 (29–54)	37.5 (21–58)	
Treatment history					
Patients on DMT- no. (%)	6 (60)	4 (40)	6 (67)	6 (50)	0.949
Pre-treatment disease duration (yr)					
Mean ± SD	9.7±10.0	8.3±5.3	11.3±6.1	8.7± 7.1	0.807
Median (Range)	7.5 (2–37)	8.0 (2–20)	8.0 (4–24)	5.5 (2–25)	
Pre-treatment relapses					

Mean ± SD	2.20±1.47	2.70±1.25	1.78±0.66	1.67±1.37	0.241
Median (Range)	2.0 (1 – 6)	2.5 (1 – 4)	2.0 (1 – 3)	1.5 (1 – 4)	
ARR	1.10	1.35	0.89	0.83	
Patients -% with ≤1 relapse	30	20	33	50	
Baseline EDSS score					
Mean ± SD	2.65±1.37	2.40±1.12	2.11±1.02	2.16±0.96	0.698
Median (Range)	3.0 (1.0–5.5)	2.5 (1.0–4.0)	2.0 (1.0–4.0)	2.0 (1.0–3.5)	

† PLP10 group

‡ Available data at Entry Baseline (n=18 for group A, n=17 for group B, n=19 for group C, n=19 for group D)

Table 2. The table section 2A reports the demographics and baseline disease characteristics for total randomized population by treatment arm.

The table section 2B reports the demographics and baseline disease characteristics of all-time on-study population by treatment arm. There were no significant between study-group differences at baseline for any characteristic.

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3A.								
Characteristics	Group A (N=10)		Group B† (N=10)		Group C (N=9)		Placebo (N=12)	
End Point	X	Y	X	Y	X	Y	X	Y
Total No. of relapses	22	17	27	8	16	13	20	25
Annual Relapse Rate (ARR)	1.10	0.85	1.35	0.40	0.88	0.72	0.83	1.04
% Reduction Compared to Placebo (primary endpoint) (Ys)¶		-18		-62		-30		N/A
P Value against placebo		0.468		0.024		0.578		
ARR change -% (Y to X)¶		-23		-70		-18		+25
P value against baseline		0.425		0.003		0.578		0.500
X: Total number of relapses of 24 months pre-treatment (baseline) Y: Total number of relapses of 24 months on-treatment ¶ Unadjusted estimate								
3B.								
Excluding patients on natalizumab	Group A (N=9)		Group B† (N=10)		Group C (N=9)		Placebo (N=10)	
End Point	X	Y	X	Y	X	Y	X	Y
Total No. of relapses	16	15	27	8	16	13	13	19
Annual Relapse Rate (ARR)	0.88	0.83	1.35	0.40	0.88	0.72	0.65	0.95
% Reduction Compared to Placebo (primary endpoint) (Ys)¶		-13		-58		-24		N/A
P Value against placebo		0.493		0.016		0.412		
ARR change -% (Y to X)¶		-6		-70		-18		+46
P value against baseline		0.857		0.003		0.578		0.354
X: Total number of relapses of 24 months pre-treatment (baseline) Y: Total number of relapses of 24 months on-treatment † PLP10 group ¶ Unadjusted estimate								
Table 3. The table section 3A reports the two year primary end points of ARR of all-time on-study population by treatment arm and percent difference with placebo. During the 24mo period on-treatment								

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3 the ARR of the group A was 0.85 with 18% decrease compared to placebo (p=0.468), of PLP10 group
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5 0.40 with 62% decrease (p=0.024), and of group C 0.72 with 30% decrease (p=0.578) and reports the
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7 comparison of the 24mo pre-treatment ARR (baseline ARR) to 24mo on-treatment ARR of all-time on-
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9 study population including patients on natalizumab.
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14 The table section 3B reports comparison of the 24mo pre-treatment ARR to 24mo on-treatment ARR of
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16 all-time on-study population excluding patients on natalizumab; and the comparison of the ARR during
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18 the 24mo period on-treatment (primary end point) between each one of the groups against placebo.
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4A.								
Characteristics	Group A (N=8)		Group B† (N=7)		Group C (N=10)		Placebo (N=7)	
	X	Y	X	Y	X	Y	X	Y
No. of Relapses	20	14	14	14	27	26	20	13
Annual Relapse Rate (ARR)	1.25	0.88	1.00	1.00	1.35	1.30	1.42	0.92
X: Total number of relapses of 24 months pre-treatment Y: Total number of relapses of 24 months on-treatment								
4B.								
Characteristics	Group A (N=20)		Group B† (N=20)		Group C (N=20)		Placebo (N=20)	
End Point	X	Y	X	Y	X	Y	X	Y
No. of Relapses	45	34	49	30	46	41	43	41
Annual Relapse Rate (ARR)	1.13	0.85	1.23	0.75	1.15	1.03	1.08	1.03
ARR Reduction -% (Y to X)¶	-25		-39		-10		-5	
P value against baseline	0.120		0.005		0.475		0.652	
% Reduction of the ARR Compared to Placebo (Ys)¶	-18		-27		0.0		N/A	
P Value against placebo	0.447		0.121		0.996			
X: Total number of relapses of 24 months pre-treatment (baseline) Y: Total number of relapses of 24 months on-treatment ¶ Unadjusted estimate † PLP10 group								

Table 4. The table section 4A reports the two year primary end point of relapses based on study design as reported by drop-out patients by Treatment Arm. The most drop-out patients that went on disease modified therapy (DMT) were from the group A and placebo with three and two patients respectively on natalizumab. These parameters justify the decreased number of relapses recorded within group A and placebo drop-outs; and this could affect the ITT analysis in favor of placebo when the total two-year recorded data are used. For PLP10 group, 14 relapses were reported at baseline and remained the same during the two-year study. For placebo, 20 relapses were reported at baseline and decreased to 13 during the two-

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3 year study. This is expected since, for PLP10 group 43% of the drop-outs were under DMT at
4 entry baseline and remained the same until the end of the study with no patient on
5 natalizumab; but 57% of the placebo group drop-outs that were under DMT at entry baseline
6 increased to 86% at the end of the study including two patients on natalizumab.
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14 The table section 4B reports comparison of 24 months pre-treatment ARR (baseline) to 24
15 months on-treatment ARR of total randomized population, by treatment arm. The ARR of
16 PLP10 group was 1.23 at baseline and 0.75 at the end of the study (39% reduction $p=0.005$),
17 and for placebo 1.08 at baseline and 1.03 at the end of the study (5% reduction $p=0.652$). No
18 statistical difference was calculated for the other two treatment arms. During the 24 months
19 on-treatment, PLP10 group presented a 27% reduction of ARR versus placebo ($p=0.121$),
20 with all groups without statistically significant results.
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Characteristics*	Group A (n=10)	Group B PLP10 (n=10)	Group C (n=9)	Placebo (n=12)	P-value Group B vs. Placebo
Annual relapse rate over 1 year**	0.80	0.40	0.78	0.83	
Total number of relapses**	8	4	7	10	
Primary end points					
Annual relapse rate over 2 years (95% CI)**	0.85	0.40 (0.15-0.87)	0.72	1.04	0.024
Total number of relapses**	17	8	13	25	
Excluding patients on natalizumab					
	(n=9)	(n=10)	(n=9)	(n=10)	
Annual relapse rate over 2 years (95% CI)	0.83	0.40 (0.10-0.79)	0.72	0.95	0.016
Total number of relapses	15	8	13	19	
Secondary end points					
Cumulative probability of sustained progression increase by 1 point on EDSS confirmed after 6 mo, over 2 years -% **	43	10 (1/10)	24	58 (7/12)	0.019
Excluding patients on natalizumab					
cumulative probability of sustained progression increase by 1 point on EDSS confirmed after 6 mo, over 2 years -%	33	10 (1/10)	24	70 (7/10)	0.006
Exploratory Results					
Patients proportion with ≤ 1 relapse over 2 years -% **	50 (5/10)	90 (9/10)	56 (5/9)	42 (5/12)	0.030
MRI					
Patients proportion with new or enlarging T2 lesions-% **	----	29 (2/7)	----	67 (4/6)	
Excluding patients on natalizumab					
Patients proportion with no new or enlarging T2 lesions-%	----	29 (2/7)	----	80 (4/5)	
DMT (interferons, glatiramer acetate) and natalizumab					
Patients proportion on DMT and natalizumab at the end of 2 years-% **	80 (8/10)†	60 (6/10)	67 (6/9)	75 (9/12) ‡	0.747
* CI denotes confidence interval.					
** Including patients on natalizumab					
† 1 out of 10 on natalizumab					
‡ 2 out of 12 on natalizumab					

Table 5. Clinical end points, according to study group for all-time on-study population.

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31
32 729 authors critically revised and approved the final version. M.C.P and I.S.P were responsible
33
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35
36 731 and the treatment physician. I.S.P and G.N.L were involved in the non-pharmacologic
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39
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27 750 No pharmaceutical companies were involved in this phase II clinical trial. The intervention is
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29 751 under a USA provisional patent; Application Number 61469081.
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34 753 Ethical approval: Cyprus National bioethics committee (reference EEBK/EII/2005/10).
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38 755 All authors have completed the Unified Competing Interest form at
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52 762 spouses, partners, or children have no financial relationships that may be relevant to the
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Article Summary

Article focus:

- The increasing prevalence of Multiple Sclerosis (MS), the limited efficacy and the side effects of the existing treatments urge the clinical need for the development of new, innovative, more effective, safe, and preventive treatment strategies.
- For the first time we propose three original nutraceutical treatment intervention-cocktails, formulated based on systems medicine through nutritional systems biology rational; with PLP10 representing the complete composition of the formulation and the other two treatments represent partial formulations.
- We have studied the proposed interventions on 80 relapsing remitting MS patients (four groups of 20 each) for a total of 42 months, in a randomized, double-blind, placebo-controlled, phase II proof-of-concept clinical trial.

Key messages:

- The per-protocol data analyses indicated that PLP10 is associated with significant efficacy **versus** placebo on both reducing the annualized relapse rate and disease progression without **any adverse or severe side effects**.
- PLP10 has probably the ability to interfere with the total known repertoire of mechanisms involved in MS pathogenesis as well as with the recovery mechanisms, neuroprotection and remyelination.; it is potentially able to manipulate global perturbed networks of the disease causing factors rather than focusing only on unique failing components.
- This proof-of-concept clinical study might be indicative and the beginning of new avenues in MS prevention and treatment and of new epoch of novel drug development with dynamic therapeutic potential for chronic complex multifactorial diseases.

Strengths and limitations of this study:

- The strength of this study is the systems medicine therapeutic approach, the length of the study the inclusion of the six months normalization (chronotherapy) period, and the study protocol following all indicated appropriate guidelines with definite inclusion/exclusion criteria and primary/secondary end points.
- The sample size and the high rate of drop-outs (palatability of the formula) are the limitations associated with the present study.
- A phase III multi-center clinical trial will establish the present intervention regime among the best choices among neurodegenerative diseases prophylaxis and MS treatment drugs faretra.

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3 919 **Figure legends**
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6 920 **Figure 1.** Omega-6 and omega-3 PUFA, their respective metabolic derivatives and their
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8 921 possible effects on inflammation.

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11 922 After consumption, the PUFAs are metabolized via several pathways (not shown) to active
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13 923 compounds that mediate inflammation and products that promote resolution of inflammation.

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17 924 Abbreviations: PL, phospholipid; IFN- γ , interferon γ ; IL-2, interleukin 2; NF κ B, nuclear
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19 925 factor kappa B; PGE₂, prostaglandin E₂; PPAR γ , peroxisome proliferator-activated receptor
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21 926 γ ; PUFAs, polyunsaturated fatty acids; TGF β , transforming growth factor β ; TNF, tumor
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23 927 necrosis factor; COX, cyclooxygenase; hydroxyeicosatetraenoic acid; HPETE,
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25 928 hydroperoxyeicosatetraenoic acid; LOX, lipoxygenase; LT, leukotriene; PG, prostaglandin;
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27 929 TX, thromboxane; RXR- γ , retinoid X receptor-gamma; Nrf2, nuclear respiratory factor;
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29 930 MMP, metalloproteinase.
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34 931 **Figure 2.** Study Flowchart

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37 932 **Figure 3.** Panel A demonstrates the ARR of all-time on-study patients during the 24mo pre-
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39 933 treatment (baseline ARR) and at different on-study intervals (6, 12, 18, 24 mo) per treatment
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41 934 arm. **

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44 935 Panel B demonstrates the ARR of all-time on-study population between 0-6, 6-12, 6-18, and
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46 936 6-24 mo period intervals, of PLP10 vs. placebo group. **

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49 937 Panel C demonstrates the ARR of all-time on-study population of PLP10 vs. placebo group at
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51 938 baseline, during 1st year, and during the 2-year on-treatment. **

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55 939 Panel D demonstrates the dispersion of relapses throughout the 2-year period of all-time on-
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57 940 study (excluding patients on natalizumab) of PLP10 (n=10) vs. placebo (n=10). Placebo
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3 941 shows an irregular dispersion of relapses in relation to PLP10 group with linear increasing
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5 942 trend while PLP10 shows a stabilized linear trend. By using the per-protocol model where
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7 943 patients on natalizumab were excluded, we could compare the number of relapses on a same
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9 944 number of patients.

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13 945 ** Including the patients on natalizumab.

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16 946 **Figure 4.** Panel 4A demonstrates the Kaplan–Meier plot of the time to sustained progression
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18 947 of disability among all-time on-study patients, excluding patients on natalizumab, receiving
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20 948 intervention A, PLP10 and C as compared with placebo. PLP10 reduced the risk of sustained
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22 949 progression of disability by 86% over two years (p=0.006). Intervention formula A reduced
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24 950 the risk of sustained progression of disability by 53% (p=0.266) and intervention formula C
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26 951 by 67% (p=0.061).

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30 952 Panel 4B demonstrates the Kaplan–Meier plot of the time to sustained progression of
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32 953 disability among ITT population receiving intervention A, PLP10 and C as compared with
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34 954 placebo. PLP10 reduced the risk of sustained progression of disability by 71% over two years
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36 955 (p=0.052, trend). Intervention formula A reduced the risk of sustained progression of
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38 956 disability by 22% (p=0.727) and intervention formula C by 40% (p=0.447).

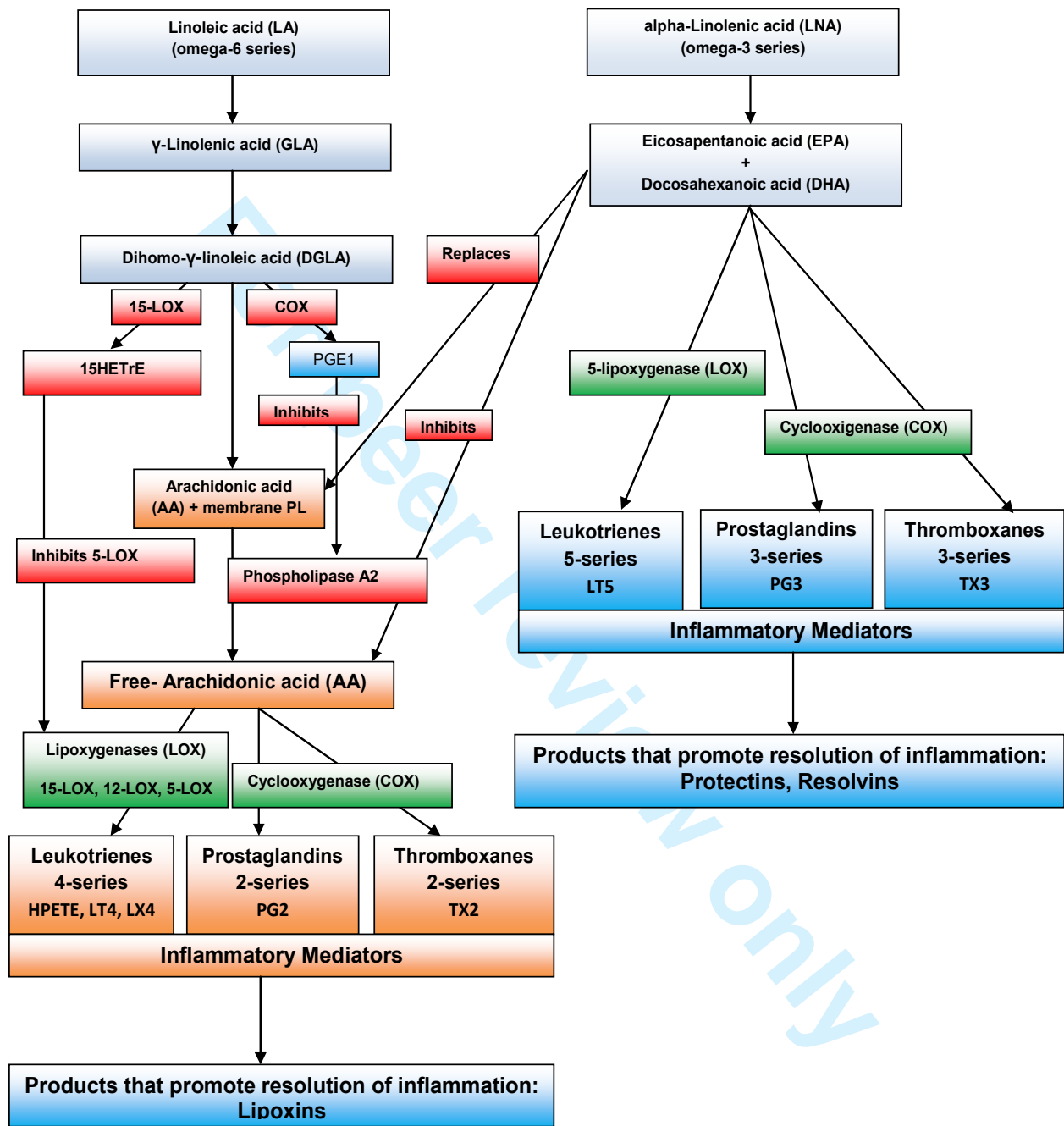
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41 957 **Figure 5.** Mean change in expanded disability status scale score as a function of visit
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43 958 number. Values are expressed as mean ± s.e.m.

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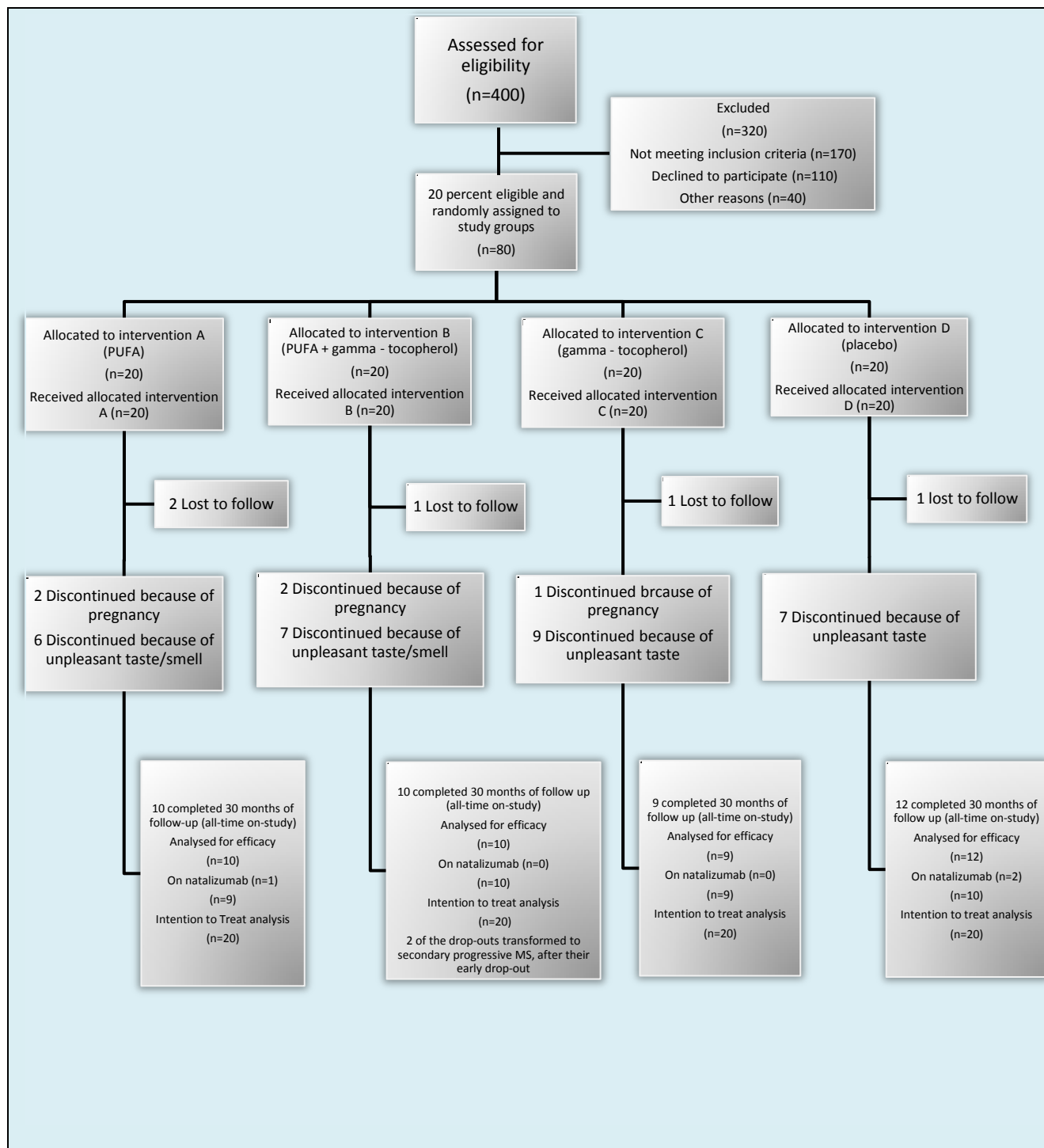
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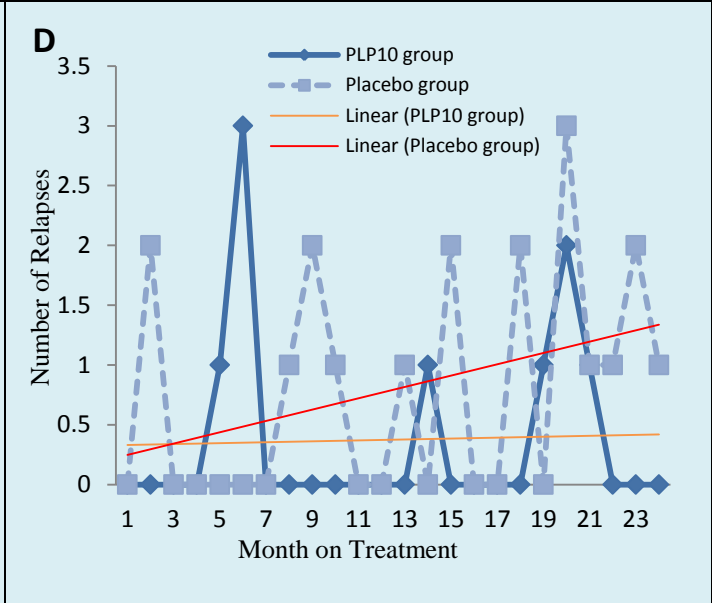
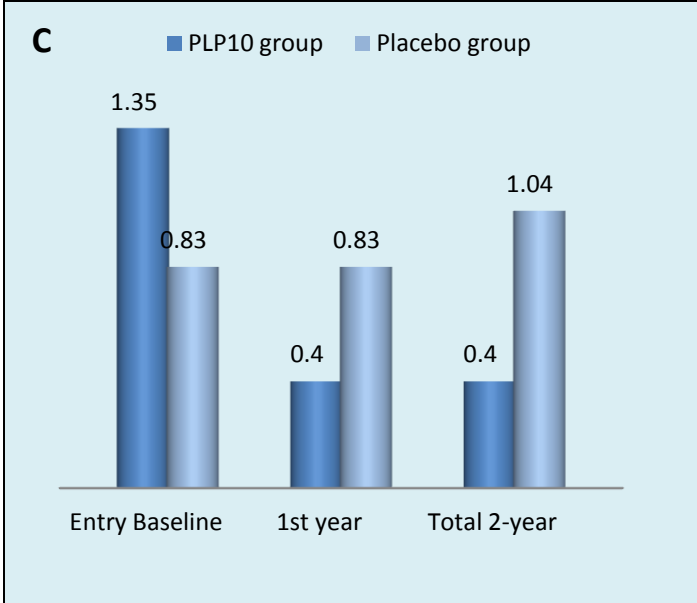
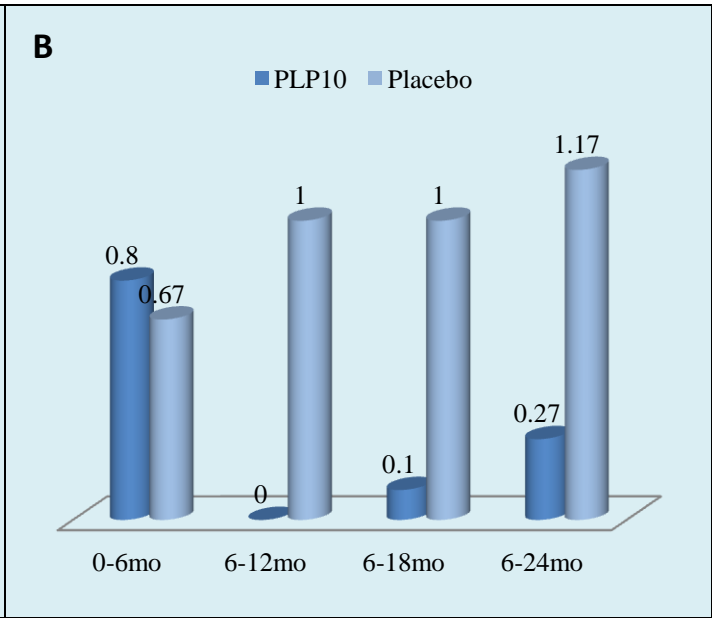
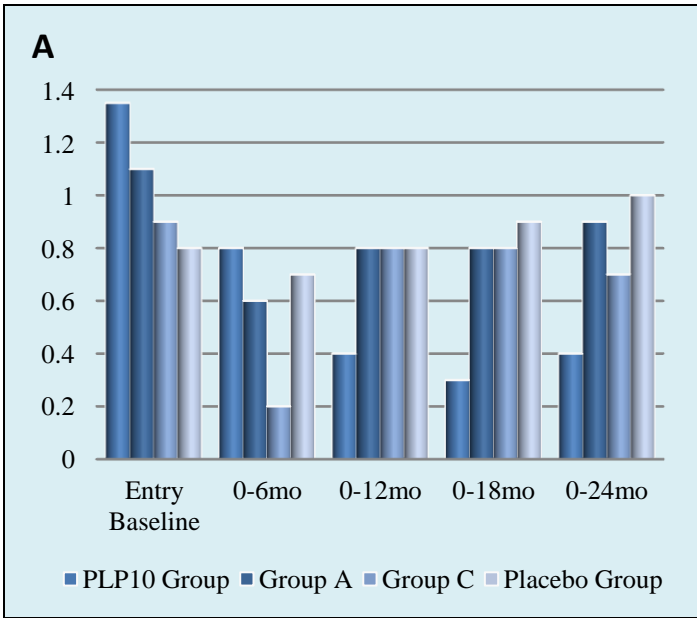
Omega-6 and omega-3 PUFA Consumption through Diet



Possible effects on inflammation:
 Reduce IFN- γ production; Reduce IL-2 production; Increase TGF β activity; Increase PGE2 activity; Inhibit arachidonic acid; Reduce TNF levels; RXR- γ and PPAR γ agonist; NF κ B expression; stimulate Nrf2; reduce MMP-2, -3, -9, and -13

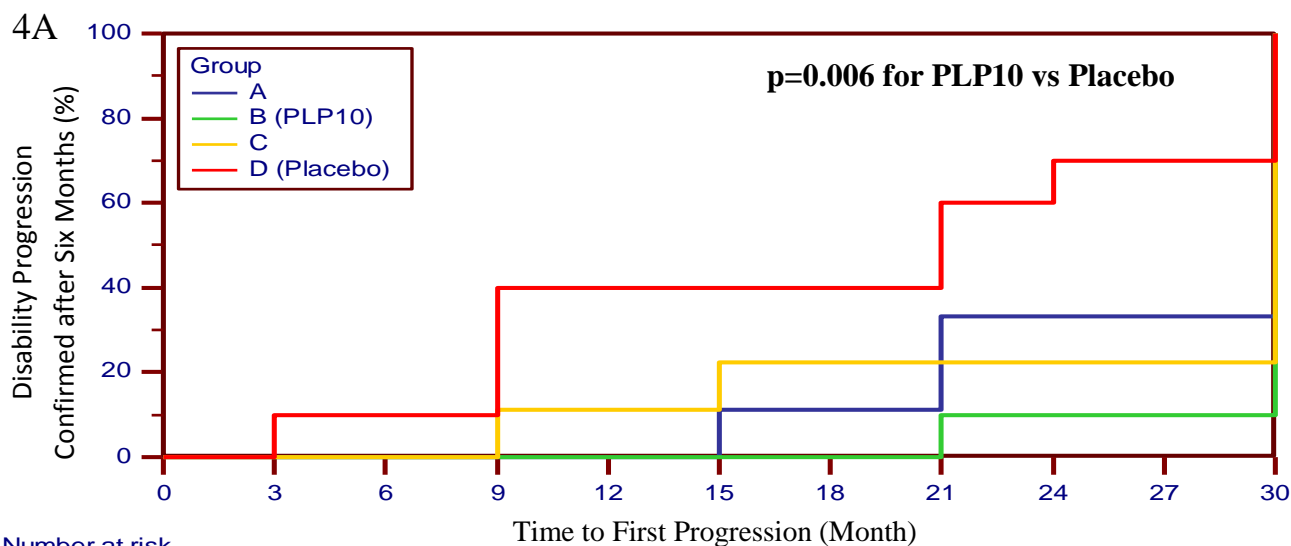


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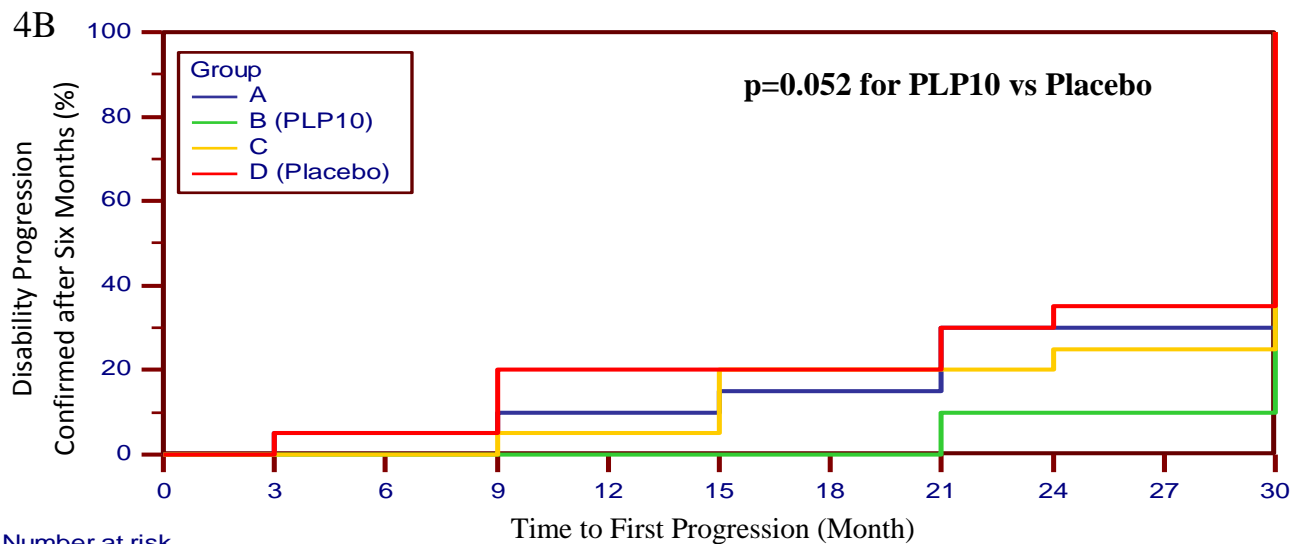
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Number at risk

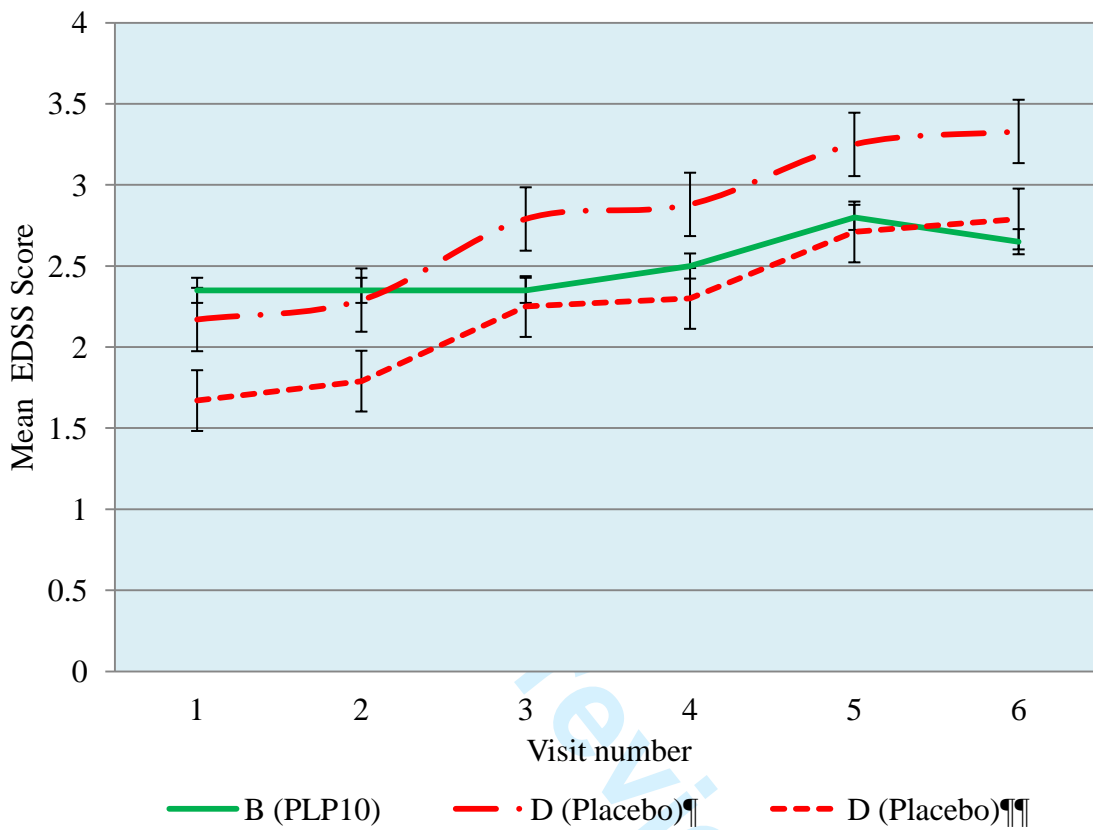
Group: A	9	9	9	9	8	8	6	6	6	6
Group: B (PLP10)	10	10	10	10	10	10	9	9	9	9
Group: C	9	9	9	8	8	7	7	7	7	7
Group: D (Placebo)	10	9	9	6	6	6	4	3	3	3



Number at risk

Group: A	20	19	19	18	18	17	17	14	14	14	14
Group: B (PLP10)	20	20	20	20	20	20	20	18	18	18	18
Group: C	20	20	20	19	19	16	16	16	15	15	15
Group: D (Placebo)	20	19	19	16	16	16	16	14	13	13	13

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3 **1 Supplementary Information**
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5 **2 Table of Content**
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Supplementary Section	Page
Supplementary Information Methods 1	2-4
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34 **Supplementary Information Methods 1**

35 **Intervention and study rational.** We are using high dosage of 1:1 omega-3:omega-6
36 polyunsaturated (PUFA) aiming to overpass and normalize global diet regional traditions and
37 habits in relation to abnormal PUFA/saturated fatty acids (SFA)/monounsaturated fatty acids
38 (MUFA) daily ratio consumption irrespective of the quantities consumed; and enough to
39 equilibrate patients' diet in line with the recommended more physiologic omega-3:omega-6
40 ratio of about 1:1-4 wt/wt as reported by Simopoulos, 2002. In addition to correct existing
41 deficiencies, cell membrane abnormalities, specifically of the immunopathological system
42 and blood mononuclear peripheral cells, and high enough for availability and immediate
43 ongoing modulation of the involved pathogenic mechanisms and network of events in MS.
44 The high dosage is also required to overpass the quantity limitations, previously discussed, of
45 diet-consumed PUFAs for cellular incorporation, especially in the central nervous system
46 (CNS) of adults. Additionally, fatty acids (FAs) must first cross the intestinal epithelium
47 before reaching the different tissues, where digestion and absorption constitute further
48 problems in their availability (Carlier, H, 1991). Omega-3 PUFA are used in re-esterified
49 form to eliminate unwanted disturbances, at the sides of action, by other fatty acids and
50 molecules present in crude fish oils but also to increase the bioavailability of the FA since
51 triglycerides have been shown to be associated with much higher bioavailability (Dyerberg et
52 al, 2010). Linoleic (LA) and gamma linolenic acid (GLA) are essential structure molecules
53 and important for any physiological (re)generation of cell membrane. GLA quantity is
54 doubled to LA to ensure high direct production of dihomo-gamma-linolenic acid (DGLA),
55 from GLA when LA cannot be metabolized, due to desaturase deficiency or malfunction.
56 Such a reduced capacity to convert LA to GLA has been associated with aging, diabetes,
57 alcoholism, atopic dermatitis, premenstrual syndrome, rheumatoid arthritis, cancer and
58 cardiovascular diseases (Bolton-Smith et al, 1997; Horrobin, 1990; Leventhal et al, 1993).
59 This is going to result in the increase of DGLA relative to arachidonic acid (AA) with DGLA
60 promoting production of prostaglandin (PG)E1 but also inhibition of phospholipase (PL)A2:
61 two major reasons and rational for their use. If other metabolic problems are involved within
62 the omega-6 series and the normal metabolites are not produced then the eicosapentaenoic
63 acid EPA available in PLP10 will substitute the function of DGLA, as a competitive inhibitor
64 of AA for PLA2. In both cases the pro-inflammatory leucotrienes, prostaglandines of the 2-
65 series (PG2) and thromboxanes of the 2 series including the platelet-activator factor (PAF)
66 will be attenuated. The synthesis of AA from DGLA by $\Delta 5$ desaturase promoted by LA/GLA
67 supplementation is very limited in humans as a result of limited activity of the enzyme
68 (Yang-Yi & Robert, 1998). AA in the body is mostly available through diet. EPA and
69 docosahexaenoic acid (DHA) are both physiologically important and crucial structured
70 molecules able to substitute excess AA and SFA within the cell membranes. EPA will
71 contribute to the inhibition (competitive to AA) of PLA2, joining the co-supplied omega-6
72 PUFA but will also participate in the production of anti-inflammatory leukotrienes,
73 prostaglandins of the 3-series (PG3) and thromboxane (TX3) along with DHA, both found in
74 the PLP10 intervention. Moreover EPA will replace AA of the membrane phospholipids and
75 both omega-3 PUFA will contribute replacing abnormal quantities of SFA and excess AA.
76 DHA is used in 3:1 ratio to EPA to cover any possible inabilities of EPA to be metabolized,
77 high enough to strongly promote high production of the aforementioned anti-inflammatory

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3 78 eicosanoids and cytokines and to be incorporated into CNS cell membranes where DHA
4 79 should be the major PUFA present, replacing other FA, probably saturated and excess of AA.
5 80 EPA, DHA, LA and GLA along with the rest of the other ingredients used (“other” omega-3
6 81 PUFA, SFA and MUFA that are usually found in the cell membranes of healthy people in
7 82 limited quantities) in the intervention regimen are for their availability as minor structural
8 83 constituents of physiological cellular membranes integrity, fluidity and overall function as
9 84 building blocks for myelin repair and/or myelination. Furthermore the PUFA used within the
10 85 cocktail intervention aimed to manipulate all other pathophysiological pathways that are
11 86 reported to be able to: as previously discussed including gene transcription for
12 87 neuroprotection and remyelination. Furthermore, PUFA are used to ensure the integrity of
13 88 blood brain barrier (BBB) and to modulate the gelatinases responsible for the T cell migration
14 89 within the CNS. Three different antioxidant vitamins (vitamin E, mostly as alpha-tocopherol,
15 90 gamma (γ)-tocopherol (vitamin E isoform) and vitamin A) are used in the regimen
16 91 preparation to support the cellular antioxidant defenses but also to protect peroxidation of the
17 92 supplied increased amounts of PUFA. Alpha-tocopherol low-molecular-weight antioxidants
18 93 will contribute to radical scavenging, interfering with gene transcription, protein expression,
19 94 enzyme activity and metal chelation (van Meeteren et al, 2005). Vitamin E (alpha tocopherol)
20 95 and vitamin A are used as antioxidants for the protection of the excess supplemented PUFA,
21 96 with alpha-tocopherol been demonstrated to protect against peroxynitrite-induced oxidative
22 97 damage, as well as able to efficiently detoxify hydroxyl, perhydroxyl and superoxide free
23 98 radicals (the elevated reactive oxygen species (ROS)); each one with different mechanism of
24 99 action, increasing the effect capability (Vatassery et al, 1998b; van Meeteren, 2005). Gamma-
25 100 tocopherol is used in high dosage since its half life is very short compared to alpha-
26 101 tocopherol and has been demonstrated to specifically protect against nitro-radicals.
27 102 Tocopherols can also exert non-antioxidant properties, including modulation of cell signaling
28 103 and immune function, regulation of transcription, and induction of apoptosis as previously
29 104 discussed (van Meeteren et al, 2005).

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39 105 PLP10 is the first preparation ever developed for MS therapy that is composed by the use of
40 106 all different previously discussed PUFA, MUFA, SFA in a cocktail preparation mixed with
41 107 the specific aforementioned antioxidant vitamins that have never been all together used
42 108 before within a specific formulation. The ingredients ratio, quality, structural form and
43 109 mostly the high dosage has never been before tested. Furthermore, the knowledge and
44 110 chronotherapy as well as other unique limitations associated with the individual molecules
45 111 used, have never been accounted, discussed, proposed or reported for any previous
46 112 therapeutic regimen.

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50 113 Through systems medicine therapeutic philosophy, by the use of PLP10, potentially MS
51 114 patients have the opportunity to be treated holistically, by natural source isolated molecules,
52 115 demonstrated as able of affecting and modulating all known pathophysiological,
53 116 immunopathological, habitual, gene related factors; thus the dynamic interconnected complex
54 117 network of events simultaneously. Possibly synergistic effects between PLP10 ingredients are
55 118 also feasible. Moreover we can speculate that treatment efficacy of PLP10, when used as

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3 119 adjunct to existing pharmaceuticals produced by reductionism, can be proven therapeutically
4 120 superior to any available treatment for MS.
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160 **Supplementary Information Methods 2**

161 **Interventions specifications.** The specific omega-3 (re-esterified glycerides) and omega-6
162 (glycerides) raw materials were purchased according to the required interventions' PUFA-
163 fraction specification (molecular structure, quantity/ratio and quality) with vitamin E (alpha-
164 tocopherol) used as antioxidant stabilizer by the supplier. The vitamins and masking aroma
165 were purchased separately. The mixing of fractions to the final required intervention-
166 composition specification was always performed by the same team of scientists under the
167 supervision of the involved medical biochemist and lipidology specialist, under appropriate
168 conditions every six months. Interventions were stored refrigerated in dark until use.

169
170 The ratios of the different ingredients used were as follows: omega-3, EPA to DHA (about 1
171 to 3 wt/wt), omega-6, LA to GLA (about 2 to 1 wt/wt), omega-3 (EPA + DHA) to omega-6
172 (LA + GLA) (about 1 to 1 wt/wt). The total omega-3 (EPA + DHA + "other" omega-3
173 PUFA) used as re-esterified triglycerol (minimum value 60%), diglyceride (about 33%),
174 monoglyceride (about 2%) structural form mixture and about 2% ethyl ester structural form,
175 with no less than 80% re-esterified triglycerol content to be DHA and EPA as a result of
176 PUFA triglycerides re-esterification of fish body oils. The "other" group of omega-3 on the
177 re-esterified glycerols included the 18:3 (alpha-linolenic acid), 18:4 (stearidonic acid), 20:4
178 (eicosatetraenoic acid) and 22:5 (docosapentaenoic acid) PUFA. The fraction of omega-6
179 (LA + GLA) used as triglycerides with no less than 50-65% triglycerol content to be LA and
180 GLA in a ratio of 2 to 1 with 18:1 (oleic acid) 14-20% and 20:1 (eicosenoic acid), 22:1
181 (docosenoic acid), 24:1 (tetracosenic acid) as additional monounsaturated fatty acids and
182 minor quantities of 16:0 (palmitic acid) 4-16%, 18:0 (stearic acid) 2-5% saturated fatty acids
183 from Borage oil source. The vitamins used were vitamin A as beta-carotene, vitamin E
184 (alpha-tocopherol) and pure gamma-tocopherol (vitamine E isoform). Citrus extract was used
185 as masking aroma and pure virgin olive oil as delivery vehicle.

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187 **The daily intervention formula agent dosages were:**

188 **Intervention formula A** daily dosage: EPA (1650mg) / DHA (4650mg) / GLA (2000mg) /
189 LA (3850mg) / total other omega-3 (600mg) / total monounsaturated fatty acids (MUFA)
190 (18:1 1300mg, 20:1 250mg, 22:1 82mg, 24:1 82mg) + total saturated fatty acids (SFA) (18:0
191 160mg, 16:0 650mg) / vitamin A (0.6mg) / vitamin E (22mg).

192 **Intervention formula B (PLP10)** daily dosage: EPA (1650mg) / DHA (4650mg) / GLA
193 (2000mg) / LA (3850mg) / total other omega-3 (600mg) / total MUFA (18:1 1300mg, 20:1
194 250mg, 22:1 82mg, 24:1 82mg) + total SFA (18:0 160mg, 16:0 650mg) / vitamin A (0.6mg) /
195 vitamin E (22mg) / gamma- tocopherol (γ -tocopherol) (760 mg).

196 **Intervention formula C** daily dosage: γ -tocopherol (760 mg) (in 16137 mg pure virgin olive
197 oil as a vehicle).

198 **Intervention formula D** daily dosage: pure virgin olive oil (16930mg).

199 Citrus aroma was added in each intervention formula to make up a total dosage of 19.5ml of
200 solution per day.

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3 201 The specific omega-3 related fraction, according to specifications required for the
4 202 interventions was prepared and purchased from EPAX AS, Aalesund, Norway; as re-
5 203 esterified glycerides from fish body oils as a source. The specific omega-6 PUFA, MUFA
6 204 and SFA related fraction, according to required specifications, was prepared and purchased
7 205 from Goerlich Pharma International GmbH, Edling, Germany, as triglycerides from Borage
8 206 seed oil (organic, cold pressed) "*Borago officinalis*" as a source. Both omega-3 and omega-6
9 207 fractions were delivered stabilized by the producer (vitamine E (alpha-tocopherol) ~ 4.5 mg/g
10 208 was used as antioxidant).

14 209 Vitamins: vitamin A as beta-carotene (HealthAid Ltd., Middlesex, United Kingdom) and pure
15 210 gamma-tocopherol (Tama Biochemical Co. Ltd., Shinjuku-ku Tokyo, Japan).

17 211 Citrus aroma (Givaudan Schwaiz AG, Dubendorf, Switzerland).

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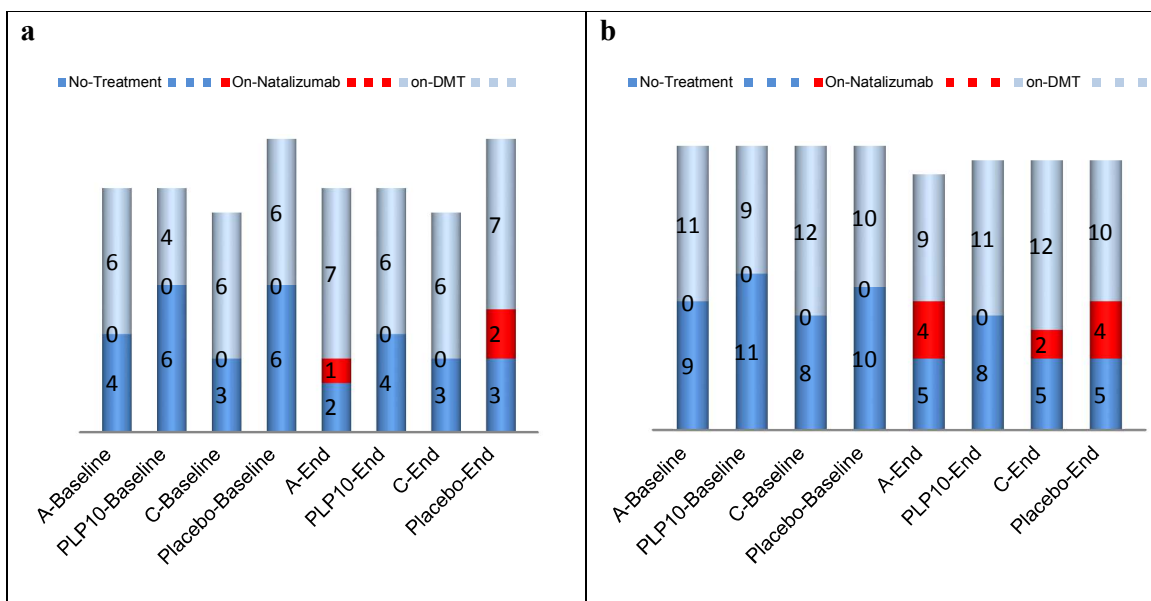
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Supplementary Information Figure 1 | Population on DMT and/or natalizumab. (a) Demonstrates the all-time on-study population per treatment arm that was receiving or not receiving DMT at entry baseline and the same population at the end of the trial (including patients on natalizumab). No statistical significant differences were calculated. The all-time on-study patients per treatment-arm that were receiving DMT at entry baseline were six patients out of ten (60%) within group A, four out of ten (40%) within PLP10 group, six out of nine (66%) within group C and six out of 12 (50%) within placebo. When the study completed, 80% of the patients in group A, 60% in PLP10 group, 66% in group C and 75% of the patients in placebo ended up on treatment. Within group A one out of eight and within placebo two out of nine patients on DMT transferred on natalizumab. (b) Demonstrates the total randomized population per treatment arm that was receiving or not receiving DMT at entry baseline and the same population at the end of the trial without lost to follow (including patients on natalizumab). A total of 61% of group A patients were on DMT at entry baseline and became 72% at the end; for PLP10 group 41% and became 53%; for group C 73% and became 74% and for placebo 53% and became 74% at study completion. At the end: for group A, four out of 13 patients on DMT transferred on natalizumab; for PLP10 group no patient was on natalizumab; for Group C two out of 14 patients on DMT transferred on natalizumab; and for placebo group four out of the 14 patients on DMT transferred on natalizumab. No significant differences measured at entry baseline between the groups.

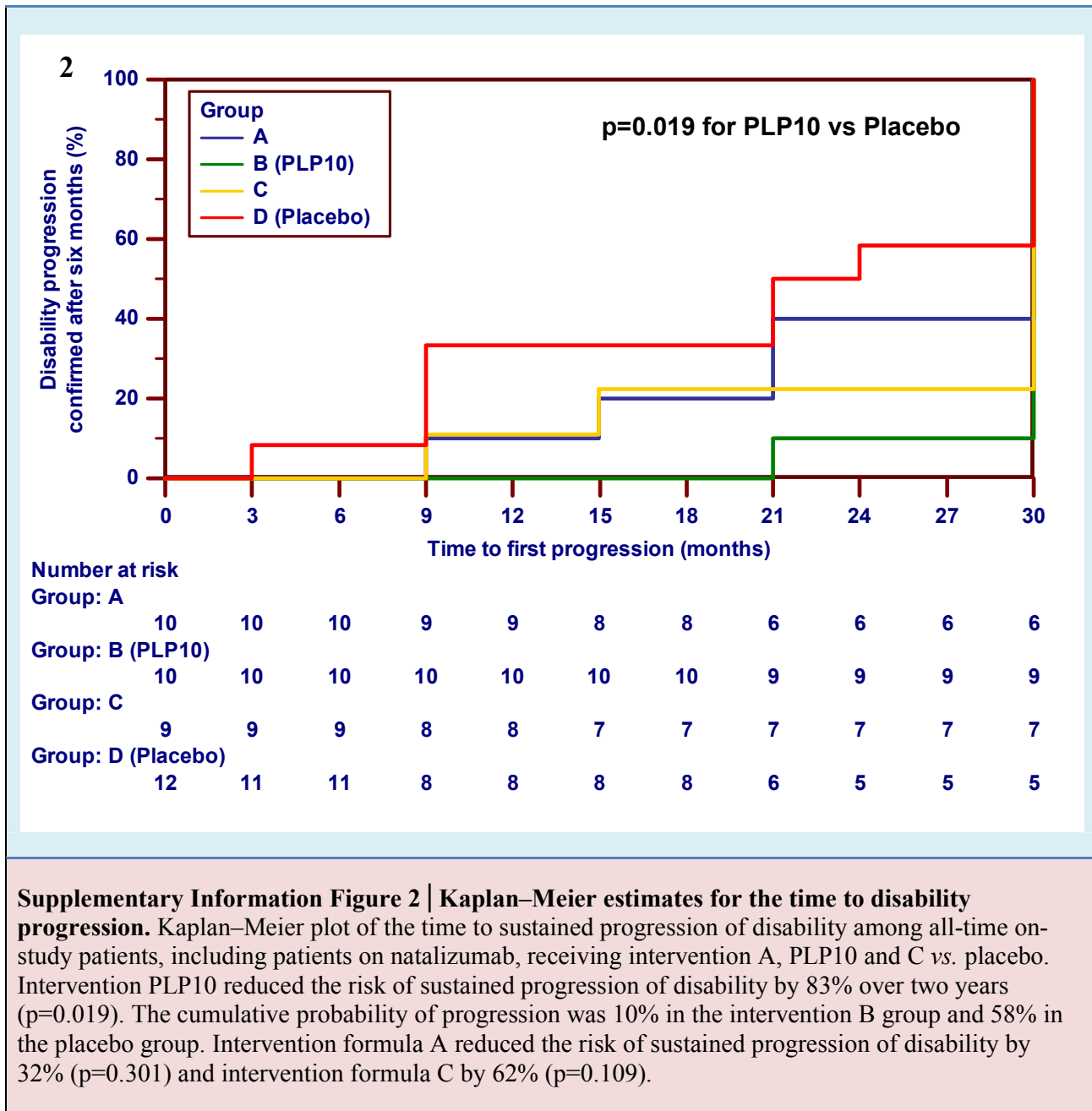
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Checklist of Items for Reporting Trials of Nonpharmacologic Treatments*

Section	Item	Standard CONSORT Description	Extension for Nonpharmacologic Trials	Reported on Page No.
Title and abstract†	1	How participants were allocated to interventions (e.g., “random allocation,” “randomized,” or “randomly assigned”)	In the abstract, description of the experimental treatment, comparator, care providers, centers, and blinding status	1,3,4
Introduction				
Background	2	Scientific background and explanation of rationale		5 to 8
Methods				
Participants†	3	Eligibility criteria for participants and the settings and locations where the data were collected	When applicable, eligibility criteria for centers and those performing the interventions	8, 13,15
Interventions†	4	Precise details of the interventions intended for each group and how and when they were actually administered	Precise details of both the experimental treatment and comparator	9,10,11, Table 1 p.28, Appendix p. 5,6
	4A		Description of the different components of the interventions and, when applicable, descriptions of the procedure for tailoring the interventions to individual participants	10, Table 1 p.28, Appendix p.5
	4B		Details of how the interventions were standardized	9,10, Table 1 p.28, Appendix p.5
	4C		Details of how adherence of care providers with the protocol was assessed or enhanced	9
Objectives	5	Specific objectives and hypotheses		7,8
Outcomes	6	Clearly defined primary and secondary outcome measures and, when applicable, any methods used to enhance the quality of measurements (e.g., multiple observations, training of assessors)		12
Sample size†	7	How sample size was determined and, when applicable, explanation of any interim analyses and stopping rules	When applicable, details of whether and how the clustering by care providers or centers was addressed	14

Randomization– sequence generation†	8	Method used to generate the random allocation sequence, including details of any restriction (e.g., blocking, stratification)	When applicable, how care providers were allocated to each trial group	9
Allocation concealment	9	Method used to implement the random allocation sequence (e.g., numbered containers or central telephone), clarifying whether the sequence was concealed until interventions were assigned		9
Implementation	10	Who generated the allocation sequence, who enrolled participants, and who assigned participants to their groups		9
Blinding (masking)†	11A	Whether or not participants, those administering the interventions, and those assessing the outcomes were blinded to group assignment	Whether or not those administering co-interventions were blinded to group assignment	9,10
	11B		If blinded, method of blinding and description of the similarity of interventions†	9,10,Appendix p.5
Statistical methods†	12	Statistical methods used to compare groups for primary outcome(s); methods for additional analyses, such as subgroup analyses and adjusted analyses	When applicable, details of whether and how the clustering by care providers or centers was addressed	13, 14, 15
Results				
Participant flow†	13	Flow of participants through each stage (a diagram is strongly recommended)--- specifically, for each group, report the numbers of participants randomly assigned, receiving intended treatment, completing the study protocol, and analyzed for the primary outcome; describe deviations from study as planned, together with reasons	The number of care providers or centers performing the intervention in each group and the number of patients treated by each care provider or in each center	15 Fig 2
Implementation of intervention†	New item		Details of the experimental treatment and comparator as they were implemented	10,15,16 Appendix p..5,
Recruitment	14	Dates defining the periods of recruitment and follow-up		11,15
Baseline data†	15	Baseline demographic and clinical characteristics of each group	When applicable, a description of care providers (case volume, qualification, expertise, etc.) and centers (volume) in each group	16,Table 2

Numbers analyzed	16	Number of participants (denominator) in each group included in each analysis and whether analysis was by “intention-to-treat”; state the results in absolute numbers when feasible (e.g., 10/20, not 50%)		15,16
Outcomes and estimation	17	For each primary and secondary outcome, a summary of results for each group and the estimated effect size and its precision (e.g., 95% confidence interval)		15 to 20
Ancillary analyses	18	Address multiplicity by reporting any other analyses performed, including subgroup analyses and adjusted analyses, indicating those prespecified and those exploratory		20
Adverse events	19	All important adverse events or side effects in each intervention group		20
Discussion				
Interpretation†	20	Interpretation of the results, taking into account study hypotheses, sources of potential bias or imprecision, and the dangers associated with multiplicity of analyses and outcomes	In addition, take into account the choice of the comparator, lack of or partial blinding, and unequal expertise of care providers or centers in each group	21
Generalizability†	21	Generalizability (external validity) of the trial findings	Generalizability (external validity) of the trial findings according to the intervention, comparators, patients, and care providers and centers involved in the trial	22
Overall evidence	22	General interpretation of the results in the context of current evidence		22 to 26

*Additions or modifications to the CONSORT checklist. CONSORT = Consolidated Standards of Reporting Trials.

†This item was modified in the 2007 revised version of the CONSORT checklist.



A novel oral nutraceutical formula (PLP10) for the treatment of relapsing remitting multiple sclerosis: a randomized, double-blind, placebo-controlled proof-of-concept clinical trial.

Journal:	<i>BMJ Open</i>
Manuscript ID:	bmjopen-2012-002170.R2
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Complete List of Authors:	Pantzaris, Marios; The Cyprus Institute of Neurology and Genetics (CING), Clinic C and PALUPA Medical Ltd Loukaides, George; The Cyprus Institute of Neurology and Genetics (CING), Neurology Clinic and PALUPA Medical Ltd Ntzani, Evangelia; University of Ioannina School of Medicine (UISM), Clinical and Molecular Epidemiology Unit, Department of Hygiene and Epidemiology Patrikios, Ioannis; The Cyprus Institute of Neurology and Genetics (CING), Clinic C and PALUPA Medical Ltd; European University Cyprus, Health Science
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Manuscripts

1 **A novel oral nutraceutical formula of omega-3 and omega-**
2 **6 fatty acids with vitamins (PLP10) in relapsing remitting**
3 **multiple sclerosis: a randomized, double-blind, placebo-**
4 **controlled proof-of-concept clinical trial**

5 **Marios C. Pantzaris***, **George N. Loukaides**, **Evangelia E. Ntzani**, **Ioannis**
6 **S. Patrikios***

7 * Both M.C.P and I.S.P are the first authors and both are the corresponding authors

8
9 The Cyprus Institute of Neurology and Genetics (CING) 1683 Nicosia, Cyprus Marios C.
10 Pantzaris Senior Consultant Neurologist Neurology Clinic C and PALUPA Medical Ltd., The
11 Cyprus Institute of Neurology and Genetics (CING) 1683 Nicosia, Cyprus George N.
12 Loukaides Clinical Dietitian/Nutritionist Neurology Clinic and PALUPA Medical Ltd.,
13 University of Ioannina School of Medicine (UISM) 45110 Ioannina, Greece Evangelia E.
14 Ntzani Assistant Professor Clinical and Molecular Epidemiology Unit, Department of
15 Hygiene and Epidemiology, PALUPA Medical Ltd at The Cyprus Institute of Neurology and
16 Genetics (CING) 1683 Nicosia, Cyprus Ioannis S. Patrikios Senior Research Scientist,
17 visiting Professor at European University Cyprus Nicosia, Cyprus, Department of Health and
18 Science. Correspondence should be addresses to e-mail: I.Patrikios@euc.ac.cy or
19 pantzari@cing.ac.cy

1
2
3 22 **Correspondence to:**
4

5 23 **Ioannis Patrikios**

6
7 24 The Cyprus Institute of Neurology and Genetics (CING)

8
9 25 Neurology Clinic C (PALUPA Medical),

10
11 26 6 International Airport Av.

12
13 27 P.O.Box 23462, 1683 Ayios Dometios. Nicosia, Cyprus

14
15 28 Tel: +357 22 358 600, +357 99 097 856;

16
17 29 i.patrikios@euc.ac.cy

18
19 30 patrikiosioannis@gmail.com
20
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31

32 **AND**

33
34 **Marios Pantzaris**

35 The Cyprus Institute of Neurology and Genetics (CING)

36 Neurology Clinic C (PALUPA Medical),

37 6 International Airport Av.

38 P.O.Box 23462, 1683 Ayios Dometios, Nicosia Cyprus

39 Tel: +357 22 358 600; +357 99 677 067

40 pantzari@cing.ac.cy
41
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42 **Keywords:** antioxidant vitamins, polyunsaturated fatty acids, multiple sclerosis, systems
43 medicine, randomized clinical trial.

45 **Word Count: 6299**

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3 **Abstract**
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5 **Objective** To assess whether our three novel interventions, formulated based on systems
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medicine therapeutic concept reduce disease activity in patients with relapsing remitting multiple sclerosis who were either treated with disease modifying treatment or untreated.

Design 30-month randomized double-blind, placebo-controlled, parallel design, phase II proof-of-concept clinical study.

Settings Cyprus Institute of Neurology and Genetics (CING)

Participants and Interventions 80 subjects were randomized into four groups of 20. The first intervention (A) was composed of omega-3 and omega-6 polyunsaturated fatty acids at 1:1 wt/wt. Specifically, the omega-3 fatty acids were docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) at 3:1 wt/wt and the omega-6 fatty acids were linoleic acid (LA) and gamma (γ)-linolenic acid (GLA) at 2:1 wt/wt. This intervention also included minor quantities of other specific polyunsaturated, monounsaturated and specific saturated fatty acids as well as vitamin A and vitamin E (alpha-tocopherol). The third intervention (C) was γ -tocopherol alone. The second intervention (PLP10) was a combination of A and C. A fourth group of 20 received a vehicle placebo. The interventions were administered per os once daily.

Main outcome measures The primary endpoint was the annualized relapse rate (ARR) of the three interventions versus placebo at two years. The secondary end point was the time to confirmed disability progression at two years.

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3 71 **Results** A total of 41 (51%) patients completed the 42-month trial. Overall, for the per-
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5 72 protocol analysis of the two-year primary end point, 8 relapses were recorded in the PLP10
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7 73 group (n=10) (0.40 ARR) versus 25 relapses in the placebo group (n=12) (1.04 ARR), a 64%
8
9 74 adjusted relative rate reduction for the PLP10 group (RRR 0.36, 95% CI 0.15 to 0.87,
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11 75 p=0.024). In a subgroup analysis that excluded patients on monoclonal antibody
12
13 76 (natalizumab) treatment, the observed adjusted RRR became stronger (72%) over the two
14
15 77 years (RRR 0.28, 95% CI 0.10 to 0.79, p=0.016). Per-protocol analysis for the secondary
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17 78 outcome at two years, time to disability progression, was significantly longer only with
18
19 79 PLP10. The cumulative probability of disability progression at two years was 10% in the
20
21 80 PLP10 group and 58% in the placebo group (unadjusted log-rank p=0.019). In a subgroup
22
23 81 analysis that excluded patients on natalizumab the cumulative probability of progression was
24
25 82 10% for the 10 patients in the PLP10 group and 70% for the 12 patients in the placebo group,
26
27 83 a relative 86% decrease in the risk of sustained progression of disability in the PLP10 group
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29 84 (unadjusted log-rank p=0.006; adjusted hazard ratio, 0.11; 95% CI 0.01 to 0.97, p=0.047). No
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31 85 adverse events were reported. Interventions A (10 patients) and C (9 patients) showed no
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33 86 significant efficacy.
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52 88 **Conclusions** In this small proof-of-concept randomized double-blind clinical trial, PLP10
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54 89 treatment significantly reduced the ARR, and the risk of sustained disability progression
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56 90 without any reported serious adverse events. Larger studies are needed to further assess the
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58 91 safety and efficacy of PLP10.
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93 **Trial registration** International Standard Randomized Controlled Trial, number
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95 ISRCTN87818535.

96 Introduction

97 Multiple sclerosis (MS) is a complex multifactorial disease that results from interplay
98 between as yet unidentified environmental factors and susceptibility genes.¹⁻³ Together, these
99 factors trigger a cascade of events, involving engagement of the immune system,
100 inflammatory injury of myelin, axons and glia, functional recovery and structural repair,
101 gliosis, and neurodegeneration.⁴ The bio-mechanisms involved are: immune-mediated
102 inflammation, oxidative stress and excitotoxicity.⁵⁻⁹ These mechanisms may all contribute to
103 oligodendrocyte and neuronal damage and even cell death, hence promoting disease
104 progression. The increasing prevalence of MS, the limited efficacy and the side effects of the
105 existing treatments urge the clinical need for the development of new, innovative, more
106 effective, safe, and preventive treatment strategies.

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108 Research has shown that multiple variables dynamically interact and many different complex
109 interrelated processes are simultaneously orchestrated for MS pathogenesis. The fundamental
110 distinctiveness of systems medicine (SM) is not just the recognition that different specific
111 complex factors are important in disease management, but that they need to be incorporated
112 in some meaningful way to treatment selection and delivery.¹⁰ The primary challenge tackled
113 by systems scientific approach is the elucidation of how these multiple variables dynamically
114 interact and how one can apply this understanding to affect the system and achieve a
115 desirable end.¹⁰ The answer might be the simultaneous interference with all involved
116 perturbed mechanisms, by using a cocktail of different specific ingredients, potentially able
117 through synergistic effect to give a long, holistic and effective treatment (Supplementary
118 Information Methods 1).

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3 120 The polyunsaturated fatty acids (PUFA) composition of membrane phospholipids plays a
4
5 121 direct role in immune and non-immune related inflammation. PUFA and antioxidant
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7 122 deficiencies along with decreased cellular antioxidant defense mechanisms have been
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9 123 reported for MS patients.¹¹The cause of these PUFA deficiencies is not entirely clear and may
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11 124 involve metabolic and nutritional alterations.¹¹
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16 126 Increased or uncontrolled inflammation contributes to several different acute and chronic
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18 127 diseases and it is characterized by the production of inflammatory cytokines, arachidonic acid
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20 128 (AA)-derived eicosanoids (prostaglandins (PGs), thromboxanes (TXs), leukotrienes (LTs),
21
22 129 and other oxidized derivatives), other inflammatory agents such as reactive oxygen species
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24 130 (ROS) , nitric oxide (NO), and adhesion molecules (Fig 2).¹² During inflammation glutamate
25
26 131 homeostasis is altered by activated immune cells releasing increased quantities of glutamate
27
28 132 that can result in over activation of glutamate receptors and in return excitotoxic
29
30 133 oligodendroglial death.^{7, 13} As such, among others, membrane-related pathology, immune-
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32 134 mediated inflammation, oxidative stress and excitotoxicity provide potentially useful
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34 135 combined targets for intervention in MS.
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41 137 *In vitro and in vivo* studies have demonstrated that dietary EPA, DHA, LA, and GLA can be
42
43 138 implicated and modulate almost all known complex network of events and pathways
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45 139 repertoire in MS pathophysiology. Brain membrane fatty acid composition can be modified
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47 140 with dietary supplementation, but the process has been showed to be age dependent (it takes
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49 141 much longer in adults versus developing brains) as well as possibly dependent on the
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51 142 quantities of the dietary/supplemented PUFAs.¹⁴ Both human and animal studies proved that
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53 143 diets high in DHA and EPA increase the proportion of these PUFA in the membranes of
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55 144 inflammatory cells and reduce the levels of AA.^{12, 15} The anti-inflammatory properties of
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3 145 omega-3 include production of PGs and TXs of the 3-series and LTs of the 5-series (Fig 2).¹⁴
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5 146 ¹⁶ Resolvins and protectins are biosynthesized from omega-3 fatty acids via cyclooxygenase-
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7 147 2/lipoxygenase (COX-2/LOX) pathways and they promote control of inflammation in neural
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9 148 tissues (Fig 2).¹⁷⁻²¹ T-cell proliferation in acute and chronic inflammation can be reduced by
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11 149 supplementation with either omega-6 or omega-3 PUFA.²² Furthermore, vitamin E is an
12
13 150 important antioxidant that can interrupt the propagation of free radical chain reactions.²³
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15 151 Vitamin E (alpha-tocopherol, an isoform of vitamin E) efficiently detoxifies hydroxyl,
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17 152 perhydroxyl and superoxide free radicals.²⁴ However γ -tocopherol (another isoform of
18
19 153 vitamin E) seems to be more efficiently implicated in trapping NO radicals.²⁵ In addition
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21 154 alpha-tocopherol exerts non-antioxidant properties, including modulation of cell signaling
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23 155 and immune function, regulation of transcription, and induction of apoptosis.²⁶
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29 157 Moreover, omega-3 fatty acids electrophilic derivatives formed by COX-2, in activated
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31 158 macrophages can stimulate the nuclear respiratory factor (Nrf2) inducing the transcription of
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33 159 neuroprotective and antioxidant related genes, and can activate the peroxisome proliferator-
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35 160 activated receptor (PPAR) γ for anti-inflammatory response.²⁷⁻²⁹ In animal studies, EPA and
36
37 161 DHA proved to be endogenous ligands of RXRs, with positive effect on neurogenesis.³⁰
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39 162 Additionally, in 2008 Salvati and coworkers reported evidense of accelerated myelination in
40
41 163 DHA- and EPA-treated animals.³² Moreover, DHA and EPA are reported to significantly
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43 164 decrease the levels of metalloproteinases (MMP) -2, -3, -9, and -13 with a significant role in
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45 165 the migration of lymphocytes into the CNS by inducing disruption of the blood brain barrier
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47 166 (BBB), an important step in the formation of MS lesions.³³⁻³⁹
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53 168 Based on the above, specific PUFA and antioxidant vitamins fulfill the criterion of biologic
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55 169 plausibility and have the potential to diminish MS symptoms severity and activity, even
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3 170 promoting recovery (remyelination).¹¹ Overall, PLP10 contains multiple ingredients (omega-
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5 171 3, omega-6 and other fatty acids and vitamins) potentially able to modulate key
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7 172 interconnected components (i.e. genes, proteins) and structural molecules (i.e. cellular
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9 173 membrane lipids, receptors) within the functional network of events of MS pathogenesis.⁴⁰

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14 175 This is a randomized phase II, single-center, double-blind, placebo-controlled, proof-of-
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16 176 concept clinical trial evaluating the therapeutic ability of PLP10 and of two other
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18 177 interventions (A and C) consisting of PLP10 constituent partial fractions (Table 1) versus
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20 178 placebo on RRMS patients.

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26 27 28 181 **Methods**

29 30 182 **Patients**

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32 183 Eligibility criteria were an age of 18 to 65; a diagnosis of RRMS according to the McDonald
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34 184 criteria; a score of 0.0 to 5.5 on the EDSS, a rating that ranges from 0 to 10, with higher
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36 185 scores indicating more severe disability; MRI showing lesions consistent with MS; and at
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38 186 least one documented clinical relapse either receiving or not disease modifying treatment
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40 187 (DMT) within the 24 months period before beginning (enrollment) of the study. Patients were
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42 188 excluded because of a recent (<30 days) relapse, prior immunosuppressant or monoclonal
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44 189 antibodies therapy, pregnancy or nursing, other severe disease compromising organ function,
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46 190 progressive MS, history of recent drug or alcohol abuse, use of any additional food
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48 191 supplement, vitamin, or any form of PUFA, history of severe allergic or anaphylactic
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50 192 reactions or known specific nutritional hypersensitivity. No monitor or limitations on
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52 193 patients' daily diet habits were included in the study design since the quantities of the
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54 194 ingredients within the formulas daily-dosage could not be significantly affected or spoiled by

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3 195 any confounding factors within any known global daily food diet (see procedures, treatment
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5 196 regimen and end-points).

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9 198 The study was conducted in accordance with the standards of the International Conference of
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11 199 Harmonization Guidelines for Good Clinical Practice. The protocol was developed by the
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13 200 investigators and it was approved by the Cyprus National Bioethics Committee and was
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15 201 overseen by an independent safety-monitoring committee evaluating the safety and over-all
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17 202 benefit-risk profiles. The adherence of care providers with the protocol was assessed by an
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19 203 external committee assigned by the funder of the project through reviews of case report
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21 204 forms. All patients gave written informed consent at the time of enrolment.
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26 27 206 **Randomization and masking**

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29 207 Patients were randomly assigned to four intervention groups in a 1:1:1:1 ratio stratified by
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31 208 gender (women to men, 3:1). Randomization was facilitated by a lottery-type pool of
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33 209 numbered balls. Patients were randomly assigned to treatment in blocks of four by flipping a
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35 210 coin as follows: for the first two drawn balls heads stratified them to the groups A/B and tails
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37 211 stratified them to the groups C/D. The other two balls were stratified accordingly. A second
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39 212 toss of the coin assigned the two patients to group A (head)/B (tail) or group C (head)/D
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41 213 (tail). The randomization scheme was generated, performed and securely stored by Helix
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43 214 Incubator Organization of Nicosia University (HIONU).
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49 216 The interventions had identical appearance and smell in dark bottles (15 daily-dose
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51 217 portions/bottle) under nitrogen bed and labeled by HIONU with code numbers, unidentifiable
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53 218 for both patients and investigators. Study data were collected by the investigators and saved
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55 219 by the HIONU that also held the blinded codes of the study. All study personnel involved in
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3 220 the conduct of the study were blinded throughout the study. Treating/examining physician,
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5 221 other investigators, pharmacist, neuroradiologist and patients were masked to treatment
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7 222 allocation.
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11 224 **Procedures and end points**

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14 225 The specific omega-3 (re-esterified glycerides) and omega-6 (glycerides) raw materials were
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16 226 purchased according to the required interventions' PUFA-fraction specification (molecular
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18 227 structure, quantity/ratio and quality) with vitamin E (alpha-tocopherol) used as antioxidant
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20 228 stabilizer by the supplier. The vitamins and masking aroma were purchased separately. The
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22 229 mixing of fractions to the final required intervention-composition specification was always
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24 230 performed by the same team of scientists under the supervision of the involved medical
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26 231 biochemist and lipidology specialist, under appropriate conditions every six months.
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28 232 Interventions were stored refrigerated in dark until use. See Table 1 and Supplementary
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30 233 Information Methods 1 and 2 for intervention specification detailed description and
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32 234 study/intervention rational.
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38 236 Participants were randomly assigned to receive: in group A, a daily dose of a 19.5ml
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40 237 mixture of EPA (1,650mg) / DHA (4,650mg) / GLA (2,000mg) / LA (3,850mg) / total other
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42 238 omega-3 (600mg) / total MUFA (1,714mg) + total SFA (18:0 160mg, 16:0 650mg) / vitamin
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44 239 A (0.6mg) / vitamin E (22mg) plus citrus-aroma (intervention A); in group B PLP10, a daily
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46 240 dose of a 19.5ml mixture of EPA (1,650mg) / DHA (4,650mg) / GLA (2,000mg) / LA
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48 241 (3,850mg) / total other omega-3 (600mg) / total MUFA (1,714mg) + total SFA (18:0 160mg,
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50 242 16:0 650mg) / vitamin A (0.6mg) / vitamin E (22mg) plus pure γ -tocopherol (760mg) plus
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52 243 citrus-aroma (intervention B); in group C, a daily dose of a 19.5ml mixture of pure γ -
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54 244 tocopherol (760mg) dispersed in pure virgin olive oil (16,137 mg) plus citrus-aroma
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3 245 (intervention C) and in group D placebo, a daily dose of a 19.5ml mixture of pure virgin
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5 246 olive oil (16,930mg) plus citrus-aroma (intervention D) (Table 1). The institution's
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7 247 pharmacist was responsible for the appropriate storage and handling of the interventions to
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9 248 the individual participants. The interventions were taken orally once daily 30 minutes before
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11 249 dinner by a dosage calibrated cup for 30 months. The ingredients, ratio and dose have been
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13 250 selected based on their biophysical interrelation to the total known multiple MS causing
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15 251 factors, their biochemical importance and the role expected to play in the normalisation and
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17 252 treatment of the involved complex network of events in the disease pathophysiology.
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19 253 Moreover, the high intake dosage was used to overcome any abnormal dietary accumulation
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21 254 of related agents as a result of patients' food intake habits, irrespective of geographical origin,
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23 255 in relation to the daily consumption ratio of the total fatty acid intake; in order to end-up with
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25 256 omega-3 to omega-6 PUFA indicated physiological body ratio composition of 1:1 wt/wt.
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32 258 The period beginning from July 1st 2007 (enrolment) until December 31st 2007 (entry
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34 259 baseline) was used for normalization period. This six-month normalization period would
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36 260 allow the interventions' agents to exert their beneficial effect (for the
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38 261 incorporation/normalization of cell membranes by oral PUFA, since they need four to six
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40 262 months to exert pivotal action on immune and neural cells, correction of antioxidant
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42 263 deficiency and body PUFA, and optimal normalization of EPA and DHA levels/ratio).⁴¹⁻⁴³
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44
45 264 The study was completed on December 31st 2009 and the recording of relapses continued
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47 265 until December 31st 2010. More clearly the study included the "normalization period" (July
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49 266 1st 2007 to Dec 31st 2007), the "on treatment" period (Jan 1st 2008 to Dec 31st 2009) and the
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51 267 12-month "extended period" (Jan 1st 2010– Dec 31st 2010).
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3 269 Depending on their clinical status and in accordance with the ethical issues governing clinical
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5 270 trials participants continued receiving the indicative regular available treatments, according to
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7 271 international guidelines with persistent evaluation of any side-effects and adverse events.
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10 272 The study was designed to end 30 months after enrolment and clinical assessments were
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12 273 scheduled at entry baseline, 3, 9, 15, 21 and 24 months on-treatment. Patients were also
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14 274 clinically examined by the treating neurologist within 48 hours after the onset of new or
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16 275 recurrent neurologic symptoms.
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21 277 The primary end point was the ARR at two years. A relapse was defined as new or recurrent
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23 278 neurologic symptoms not associated with fever or infection that lasted for at least 24 hours
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25 279 and accompanied by new neurologic signs. Relapses were treated with methyl-prednisolone
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27 280 at a dose of 1g intravenous per day, for three days followed by prednisone orally at a dose of
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29 281 1mg/kg of weight per day on a tapering scheme for three weeks. The secondary end point at
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31 282 two years was the time to confirmed disability progression, defined as an increase of 1.0 or
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33 283 more on EDSS, confirmed after six months (progression could not be confirmed during a
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35 284 relapse). The final EDSS score was confirmed six months after the end of the study. A post-
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37 285 hoc analysis was performed assessing the proportion of patients free from new or enlarging
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39 286 T2 lesions on brain MRI scans at the end of the study for the per-protocol participants of the
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41 287 group receiving the highest effective intervention versus placebo. Comparison was made only
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43 288 versus the available archival MRI scans up to three months before the enrolment date. MRI
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45 289 scans were performed and blindly analyzed at an MRI evaluation centre. The patients
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47 290 continued to be followed for additional 12 months after completion of the trial and relapses
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49 291 were recorded. Finally, patients were strongly encouraged to remain in the study for follow-
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51 292 up assessments even if they had discontinued the study drug.
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3 294 Blood samples were collected from all randomized patients at the time of enrolment, at every
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5 295 scheduled clinical assessment and during relapses. To check individual compliance with
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7 296 intake, the fatty acids composition of patients' red blood cells' membranes was determined,
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10 297 by gas chromatography, according to a standard protocol. The fatty acid analyses were
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12 298 performed after study termination and thus did not influence the blinding.

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16 300 The involved neurologist was experienced with more than 20 years in practice and trained to
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18 301 standardise EDSS scoring procedures, examined patients, made all medical decisions,
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20 302 determined the EDSS score and reviewed adverse or side-effects. The medical biochemist,
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22 303 specialist on lipidology and immunology and the registered clinical dietitian, members of the
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24 304 investigator team were experienced with more than 25 years in practice. Patients were able to
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26 305 contact the neurologist at any time if there was any adverse event, side-effect or allergic
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28 306 reaction. The study drug was not suspected to have any clinical or laboratory adverse effects
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30 307 different from placebo that could disturb the double-blind nature of the trial. Therefore, the
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32 308 same study-neurologist functioned as both the treating and evaluating physician.

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36 310 Safety measures were assessed from the time of enrollment until 12 months following study
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38 311 completion. Haematological and biochemical tests were performed at enrolment and at every
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40 312 12 months, including full blood count, renal and liver function tests, proteins, cholesterol,
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42 313 triglycerides, glucose and electrolytes.

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46 315 The whole procedure followed the clinical trial guidelines as required by the USA Food and
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48 316 Drug Administration, European Medicines Agency, and the Committee for Medicinal
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50 317 Products for Human Use.⁴⁴

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3 319 **Statistical analysis**
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5 320 Power calculations could not be done before the study because of the lack of information
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7 321 from previous studies on potential effect sizes. In 2005 the prevalence of MS in Cyprus
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9 322 (600,000 population) was 120/100.000. Based on the aforementioned MS patients' numbers
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11 323 of our country and the centre of reference, the CING, we were able to enrol the 20% of the
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13 324 total RRMS patients eligible for treatment in the trial. Sample size was strictly based on this
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15 325 subjects' availability parameter and the novelty of the assessed intervention.
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21 327 Baseline characteristics were compared across all intervention groups by ANOVA or
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23 328 Kruskal-Wallis rank test for continuous variables and by mean Fisher's exact test for
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25 329 categorical variables, as appropriate.
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30 331 For the primary outcome, the ARR was analysed in a pair-wise fashion for the active
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32 332 interventions compared to placebo using negative binomial regression models adjusted for
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34 333 number of relapses within two years before baseline, EDSS score at baseline and DMT. The
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36 334 relapse rate was calculated as the total number of relapses divided by the total number of
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38 335 patient-years followed for each treatment group. ARR differences were also calculated
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40 336 among all comparable parameters and reported as percent difference.
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45 338 For the secondary end-point outcome, the time to disability progression, Kaplan–Meier
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47 339 curves were constructed. Progression to disability and time thereof was compared in a pair-
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49 340 wise fashion for the active interventions versus placebo by the log-rank test in the main
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51 341 analysis and by Cox proportional-hazards models with adjustment for baseline EDSS score,
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53 342 age and DMT in the supportive analysis. Each test was performed with a significance level of
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3 343 0.05. Multivariate models considered all variables with $P < 0.1$ on univariate models. There
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5 344 was no overt violation of the proportionality assumption.
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10 346 Both, per-protocol and intention to treat (ITT) analyses were performed, for different sets of
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12 347 research questions to be answered, and both are reported. Missing data of the five lost to
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14 348 follow patients were imputed by use of the last-observation-carried-forward (LOCF)
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16 349 approach. Due to the proof-of-concept design of the study, the considerable non-adherence
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18 350 rate (49%) and the interpretation issues caused thereof regarding the ITT analysis, the per-
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20 351 protocol analysis considered being more informative and appropriate method approach to
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22 352 answer the research addressed questions of efficacy of the interventions when subjects were
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24 353 continuously following the protocol. All statistical analyses were well defined a priori. All
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26 354 analyses were performed with STATA SE 10.0 (College Station, TX, USA). P-values are
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28 355 two-tailed.
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33 34 357 **Role of the funding source**

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36 358 The funders had no role in study design, data collection and analysis, decision to publish, or
37
38 359 preparation of the manuscript. All members of the writing group had full access to all study
39
40 360 data and contributed to its interpretation and prepared, reviewed, and approved the
41
42 361 manuscript for submission. All authors had final responsibility for the decision to submit the
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44 362 paper for publication.
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49 50 364 **Results**

51 52 365 **Study population**

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3 366 From July 2007 through December 2010 (including the 12-month extended period), a total of
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5 367 80 MS patients were randomly assigned to a study group at the CING (tertiary neurological
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7 368 center).
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11 370 Among the 80 patients, 20 patients were randomly assigned to each of the three groups to
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13 371 receive the interventions and 20 to receive placebo (Fig 1). Baseline characteristics of both
14
15 372 the ITT and the per-protocol populations were similar across groups (Table 2A and 2B). All
16
17 373 patients that drop-out completed follow-up until study completion and were included in the
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19 374 ITT analyses (Table 4). Five patients were lost to follow before their first scheduled visit and
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21 375 two other patients that dropped-out before their first scheduled visit progressed to secondary
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23 376 progressive MS. Fifteen patients dropped-out without successfully completing the
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25 377 “normalization” period including five pregnancies. Another 17 patients dropped-out early
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27 378 after entry baseline. Seven patients that dropped out were given monoclonal antibody
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29 379 treatment (natalizumab). Overall, a total of 41 (51%) patients completed the 42-month study
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31 380 (July 2007 through December 31st 2010, including the 12-month extended period) where one
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33 381 patient from group A and two from the placebo group transferred on natalizumab, and 39
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35 382 (49%) patients either withdrew (drop-out) or lost to follow. Reasons for study-interventions
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37 383 discontinuation are listed in Figure 2.
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44 385 **Efficacy**

45 386 **Relapses**

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47 387 As a proof-of-concept trial we primarily needed to answer whether the interventions were
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49 388 effective for those MS patients who adhere to the assigned treatment, the per-protocol
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51 389 analysis.⁴⁵ For the sake of methodological comprehensiveness we also present the ITT
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53 390 analysis as a secondary analysis, to answer a different question, complementary to our core
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3 391 hypothesis; like what happened to MS patients who were placed on the interventions (the
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5 392 effect of assignment).⁴⁵
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10 394 Regarding the per-protocol analysis, during the first year of treatment, the ARR was 0.80,
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12 395 0.40, 0.78 and 0.83 for the four intervention groups respectively. During the second year, the
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14 396 ARR was 0.90, 0.40, 0.67 and 1.25 for the four intervention groups respectively. Overall, for
15
16 397 the two-year primary end point, 8 relapses were recorded for the 10 patients in PLP10 group
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18 398 (0.40 ARR) versus 25 relapses for the 12 patients in placebo (1.04 ARR), a 64% adjusted
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20
21 399 relative rate reduction (RRR) for the PLP10 group (RRR 0.36, 95% confidence interval (CI)
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23 400 0.15 to 0.87, $p=0.024$) (Tables 3A, 5 and Fig 3A and 3C). Excluding patients on monoclonal
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25 401 antibody (natalizumab) treatment, the observed adjusted RRR became stronger (72%) over
26
27 402 the two years (RRR 0.28, 95% CI 0.10 to 0.79, $p=0.016$, Tables 3B and 5). Pair-wise
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29 403 comparisons for the other two groups against placebo did not yield statistically significant
30
31 404 results (Tables 3A, 3B). The proportion of patients with ≤ 1 relapse for the two years on-study
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33 405 was higher in the PLP10 group than in the placebo group (90% versus 42%, $p=0.030$, Table
34
35 406 5). Seeking to investigate further the observed difference, we compared the relapse rate
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37 407 during the 24 months before entry to the study to the 24 months on-treatment for each
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39 408 intervention group. We observed a statistically significant relative reduction in the ARR
40
41 409 (70%) only in the PLP10 group (RRR 0.30; 95% CI 0.14 to 0.65, $p=0.003$, Table 3A);
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43 410 within-group comparisons for the three other groups ARR reduction was not significant and
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45 411 remained not significant when natalizumab treated patients were further excluded from the
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47 412 analysis. The effect of PLP10 through time at different time-windows versus placebo for all-
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49 413 time on-study patients is shown in Figures 3A to 3D. The ARR analysis, within time-
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51 414 windows, was not an assigned endpoint, but it could help in the process of evaluating parallel
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53 415 information as the time needed for a specific treatment intervention activity to be evident, as
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3 416 well as the efficacy profile through time. PLP10 reached maximum effect within a year on-
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5 417 treatment (counting from the entry baseline) and remained stable at an ARR of 0.4,
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7 418 displaying a steadily reduced ARR with long free-relapse time-windows. These group B
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9 419 characteristics are considered important parameters of a successful MS treatment where the
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11 420 rule than the exception is the heterogeneity among patients' disease evolution. Specifically,
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13 421 Figure 3D demonstrates the dispersion of relapses throughout the 2-year period of all-time
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15 422 on-study (excluding patients on natalizumab) of PLP10 (n=10) versus placebo (n=10).
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17 423 Placebo, in line with the existing knowledge of how relapse history works in relation to future
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19 424 relapses on MS patients (contagion phenomenon) indicates the expected linearly increased
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21 425 trend of the relapse incidence.⁴⁶ The same phenomenon was true for the groups A and C.
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23 426 Finally, during the 12 month post-study extended period (January 1st 2010 to December 31st
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25 427 2010) all-time on-study patients that received PLP10, showed persistent benefit in the ARR
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27 428 compared to placebo (six relapses for the 10 subjects within PLP10, 0.6 ARR versus 19 for
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29 429 the 12 subjects within placebo group, 1.58 ARR) indicating a statistically significant 62%
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31 430 adjusted relative rate reduction in the ARR for the PLP10 group (RRR 0.38, 95% CI 0.12 to
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33 431 0.99, p=0.046).
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38 433 Regarding the ITT analysis, within PLP10 group, none of the nine drop-out patients changed
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40 434 to natalizumab and two out of nine discontinued because of pregnancy, whereas two out of
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42 435 seven drop-out patients from the placebo group changed to natalizumab (a total of four
43
44 436 patients within the placebo arm population were on natalizumab, including the two patients
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46 437 that transferred while all-time on-study versus none within PLP10 group (Supplementary
47
48 438 Information Fig 1). As reported, natalizumab reduces ARR by 68%, decreases the possibility
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50 439 of disability progression by 43% with 57% of patients free of new or enlarging T2 lesions on
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52 440 MRI scans compared to 15% on placebo.⁴⁷ The relapses of the drop-out patients are reported
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3 441 in Table 4A. As expected no statistically significant differences in the ARR were calculated
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5 442 for the comparison of any group versus placebo for the 24 months on-treatment, with a 0.75
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7 443 ARR within PLP10 (30 relapses) and 1.03 ARR within placebo (41 relapses) group, a 27%
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9 444 ARR reduction (Table 4B). Interestingly, despite the high non-adherence rate, there was a
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11 445 statistically significant difference for the comparison of the ARR in the 24 months before
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13 446 entry baseline to the 24 months on-treatment for the PLP10 group (RRR 0.45, 95% CI 0.26 to
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15 447 0.78, $p=0.005$).
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20 21 449 **Disability progression**

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23 450 Regarding the per-protocol analysis, at two years, the time to disability progression, with
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25 451 confirmation after six months (secondary end-point) was significantly longer only with
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27 452 PLP10. The cumulative probability of disability progression was 10% in the PLP10 group
28
29 453 and 58% in the placebo group ($p=0.019$) (Supplementary Information Fig 2). After excluding
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31 454 patients on natalizumab, there was an increased statistically significant difference between
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33 455 the PLP10 and the placebo group for the same analysis ($p=0.006$) (Fig 4A). At two years, the
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35 456 cumulative probability of progression was 10% in the PLP10 group and 70% in the placebo
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37 457 group, which represents a decrease of 60 percentage points or a relative 86% decrease in the
38
39 458 risk of sustained progression of disability within PLP10 group (adjusted hazard ratio, 0.11;
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41 459 95% CI 0.01 to 0.97, $p=0.047$). One versus seven out of ten patients progressed to confirmed
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43 460 disability in the PLP10 and the placebo groups respectively when patients on natalizumab
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45 461 were excluded. No statistically significant difference was observed for any comparison of the
46
47 462 other two groups compared to placebo (Fig 4A and Supplementary Information Fig 2).
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54 464 Regarding the ITT analysis, at two years, the cumulative probability of progression was 10%
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56 465 in the PLP10 group and 35% in the placebo group ($p=0.052$, a trend for an effect), which
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3 466 represents a decrease of 25 percentage points or a relative 71% decrease of the PLP10 for the
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5 467 risk of sustained progression of disability (adjusted hazard ratio 0.22, 95% CI 0.04 to 1.07,
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7 468 $p=0.06$) (Fig 4B). Two versus seven out of the total randomized patients progressed to
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9 469 confirmed disability in the PLP10 and the placebo groups respectively. No significant
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11 470 differences were observed for groups A or C against placebo (Fig 4B). The mean change in
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13 471 Expanded Disability Status Scale (EDSS) score as a function of visit number is shown in
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15 472 Figure 5.
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21 474 **MRI**

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23 475 Over two years, the MRI results support the overall conclusion from the study that PLP10 has
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25 476 a positive effect on disease activity since only 29% from the PLP10 group as opposed to 67%
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27 477 from the placebo group developed new or enlarging T2 lesions (57% relative risk reduction).
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29 478 Excluding patients on natalizumab there is an increased relative risk reduction (64%) between
30
31 479 PLP10 as opposed to placebo with 29% of patients on PLP10 and 80% on placebo with
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33 480 development of new or enlarging T2 lesions (Table 5).
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38 482 **Safety**

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40 483 Over the course of the 30 month study no significant adverse events were reported from any
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42 484 group. According to a questioner procedure the only aetiology for drop-outs was the
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44 485 palatability and smell of the formula preparations. Nausea was reported by two patients. No
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46 486 abnormal values observed on any of the biochemical and haematological blood tests. No
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48 487 allergic reactions reported.
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54 489 **Discussion**

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3 490 In this proof-of-concept clinical trial assessing the safety and efficacy of three cocktail
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5 491 intervention formulas in RRMS, we observed a significant benefit for the novel PLP10
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7 492 intervention compared to placebo for both the ARR and the progression to disability. Our
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9 493 results include analyses pertaining to a total of 42 months study collected data, including the
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11 494 12-month, free of intervention treatment, extension period. We focused on the per-protocol
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13 495 data analysis since it is the appropriate method to best provide the answer to the proof-of-
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15 496 concept trial-addressed question. The high drop-out rate was mostly the result of formulas
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17 497 palatability, a common phenomenon in trials using oily interventions where a lot of patients
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19 498 tend to drop-out soon after first dosage. We thus present our main per-protocol analysis, as
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21 499 well as a subgroup analysis excluding patients on natalizumab. We have found a statistically
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23 500 significant reduction in the ARR and the disability progression comparing not only patients
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25 501 on PLP10 versus placebo but also comparing the ARR of the PLP10 patients in the 24-month
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27 502 period prior to the study to the ARR of the 24 months on-study; the observed differences
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29 503 became larger when patients that received natalizumab (the most potent disease modifier)
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31 504 were excluded. The ARR decreased within a year on PLP10 and significantly remained stable
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33 505 until study completion. Statistically significant difference of ARR between patients on PLP10
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35 506 versus placebo continued for the additional 12 month extended period (persistent effect)
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37 507 without significant difference on DMT. These clinical findings are supported by the results
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39 508 regarding the MRI analysis where the proportion of patients free from new or enlarging brain
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41 509 T2 lesions was also higher in PLP10 group versus placebo. The persistent effect within the
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43 510 extended period it is considered of major importance and supportive of the results since it is
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45 511 in agreement with the very long washouts, reported necessary, for omega-3 fatty acids and
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47 512 especially DHA to return towards pretreatment values within the fatty acids of plasma,
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49 513 platelets, monocytes and red blood cells.⁴² This study also provides important 30-month,
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3 514 placebo-controlled information about the safety of PLP10, A and C interventions. No severe
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5 515 side effects have been reported.
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10 517 As medications used to treat MS become increasingly highly specific and potent, attention to
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12 518 safety is paramount. Current available treatments are products of reductionism, partially
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14 519 effective, associated with severe side effects without (re)myelinating or neuroprotective
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16 520 abilities. Interferons and glatiramer acetate, the most widely used first-line MS drugs
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18 521 available today, are associated with the least severe side-effects among MS therapies but they
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20 522 are reported with only 29-33% ARR reduction and with no significant effects on the
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22 523 progression of disability. Natalizumab as previously discussed and Fingolimod with 54%
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24 524 ARR reduction (without significant benefit on the progression of disability) are second-line
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26 525 drugs associated with severe side-effects.^{47, 48}
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32 527 No existing MS treatment has ever been designed as a result of SM concept approach or with
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34 528 a potential to effectively stimulate intrinsic remyelinating and neuroprotecting mechanisms or
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36 529 exert such an action. Now we propose that a holistic SM model approach has to be applied by
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38 530 synchronized action on all involved perturbed mechanisms. PLP10 has innovative
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40 531 characteristics with a postulated efficacy attained through different mechanisms of action and
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42 532 probably by the synergistic effect of its constituent ingredients. PLP10 has all the
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44 533 characteristics of a medical food with the action to feed a normal metabolic process by
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46 534 supplying nutritional structural membrane precursors, building blocks, and vitamins from
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48 535 dietary sources that enhance remyelination and neuroprotection and simultaneously promote
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50 536 normalization of all cellular membranes lipid content. The intention is to normalize the
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52 537 specific nutritional requirements of the MS patients.
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3 539 Different factors and molecular entities appear to be part of the possible aetiology for MS
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5 540 with specific PUFA and antioxidants found to be key substances related to all known
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7 541 pathogenic and recovery mechanisms. But, it is well established that MS patients are
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9 542 characterized with significant deficiencies in antioxidant efficiency as well as in PUFA levels
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11 543 in blood and cellular membranes.^{11, 49-51}
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16 545 According to one hypothesis, the change in the ratio of omega-3 to omega-6, due to
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18 546 increasing consumption of omega-6 PUFA, meaning high accumulation of AA, in the
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20 547 Western diet, may be one of the major factors responsible for the increasing incidence of
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22 548 inflammatory diseases relative to populations. In the Western diet, the ratio of omega-3 to
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24 549 omega-6 is about 1:20–30; in populations that consume fish-based diets, the ratio is about
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26 550 1:1–2.^{52, 53} The intervention daily dose was aiming and believed to be high enough to
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28 551 restore/amplify body efficient antioxidant activity and ensure cellular membranes lipid profile
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30 552 normalization (PUFA content) and simultaneously potentiate involvement of the ingredients
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32 553 in the anti-inflammatory and recovery mechanisms. Diet fatty acid molecules need about six
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34 554 months period to exert their beneficial effect and this essential parameter was for the first
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36 555 time under consideration in our study design (normalization period).⁴² This chronotherapy
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38 556 parameter it is of major importance in line with the SM treatment philosophy and if it is not
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40 557 included in the trial design the possibility of misleading result evaluation greatly increases. In
41
42 558 fact, considering that omega-3 supplementation can release and replace excess AA within the
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44 559 cellular membranes, we can speculate that an increased inflammatory activity can possibly
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46 560 result during the first six months of supplementation (during normalization period).
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54 562 The maintenance of myelin requires continued turnover of its components throughout life.^{54,55}
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56 563 In addition to the EPA, DHA, LA, and GLA, PLP10 was containing limited quantities of
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3 564 other structural PUFA, specific MUFA (mostly oleic acid) and SFA (palmitic and stearic
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5 565 acid), specifically to provide a direct source for neuronal cell membranes rehabilitation and
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7 566 for (re)myelination and neuroprotection since they are all major components, precursors and
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10 567 building blocks of any new physiological myelin and cellular membranes in general.
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12 568 Assembly of the correct molecules into myelin membrane may be especially critical during
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14 569 active synthesis. Possibly, if critical constituents aren't available or are metabolically
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16 570 blocked, amyelination, dysmyelination or demyelination may ensue.⁵⁶
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21 572 The well known and established safety of the ingredients used and the protocol guidelines
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23 573 were supportive reasons for us to proceed with the clinical study even though with limitation
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25 574 on the pre-estimation of required trial sample size as it was discussed in method section. The
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27 575 adherence of the subjects is another issue but the duration of the study (42 months) is adding
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29 576 power to the results;⁴⁴ having the research questions been consciously and carefully
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31 577 approached and answered. Furthermore, the statistical methodologies used along with the
32
33 578 appropriate adjustments, broadly accepted for MS clinical trials, power the findings, results,
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35 579 and significance. The baseline characteristics of the treatment arms could possibly be
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37 580 considered indicative of four very active groups of patients but that was the result of the
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39 581 limited number of RRMS population eligible for the study within Cyprus. On the other hand
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41 582 the balanced baseline characteristics without statistical differences, the statistical adjustments
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43 583 (for all important baseline parameters i.e. relapses, EDSS, age and DMT) and the
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45 584 randomization within four different groups are the safety valves against data
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47 585 misinterpretation. Yet, in small randomized control trials with a high drop-out rate, the per-
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49 586 protocol analysis could be affected by the characteristics of the patients dropping out. In
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51 587 order to safeguard our findings in the best possible way under the circumstances, we
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53 588 proceeded to adjusting for confounders. Moreover, we cannot discard our finding as a false
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3 589 positive, given that this is a randomized, double-blind, placebo-controlled clinical trial and,
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5 590 despite its small sample size, represents a piece of evidence that only a larger randomized
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7 591 controlled trial can replicate or refute. It is possible to question why DMTs efficacy cannot
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9 592 be emerged out of the data analysis, of the four treatment arms, and in accordance to their
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11 593 published values. We believe that the limited efficacy of the DMTs, the sample size and the
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13 594 statistical adjustments were strong limiting determining factors for such an indication to be
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15 595 countable. An additional argument is that the efficacy reported for the analysis of pre-
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17 596 treatment (24 months before entry baseline) versus on-trial ARR could be considered as
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19 597 potentially biased due to differences of how relapses were defined during the course of a
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21 598 study compared to pre-treatment period; or due to regression to the mean or placebo effect.
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23 599 This analysis was performed as an additional exploratory analysis that we were able to do due
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25 600 to the availability of data. The relapses of the two pre-treatment years were drawn out of the
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27 601 patients' archival records by the same treating neurologist involved in the study (MP), and
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29 602 according to the patients' hospitalization date for receiving intravenous methyl-prednisolone.
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31 603 This analysis was not used as a primary or a secondary end-point under investigation
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33 604 although it is usually reported by many clinical studies. As a matter of fact many early phase
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35 605 trials are based only on such an analysis (before versus after treatment results). In almost all
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37 606 MS trials the number of relapses within the two years before baseline is a factor under
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39 607 adjustment for the statistical analyses.⁴⁸ The inclusion of the post-hoc MRI analysis is another
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41 608 limiting factor that needs attention since it was used as an additional aside exploratory
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43 609 approach (due to study budget limitations it was not possible to be used as a formal
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45 610 endpoint); but the MRI evaluation was blinded and can be considered as representative of the
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47 611 randomized subjects within the treatment arms. As far as the regression to the mean and the
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49 612 placebo effect concerns we believe that the 6-month normalization period is an accountable
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51 613 and valuable eliminating factor of the possible effect; as well as the presence of four groups,
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3 614 where only the PLP10 treatment arm is associated with statistically significant efficacy versus
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5 615 placebo.

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9 617 Our observations are consistent with the idea that simultaneous availability of specific PUFA
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11 618 along with other major membrane and myelin building blocks in combination with specific
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13 619 antioxidants, within optimum quantity, quality, ratio and structural form can possibly result to
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15 620 a more appropriate holistic therapy reducing MS disease activity. This is probably succeeded
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17 621 through synergistic and/or simultaneous effect on the interactions and dynamics of the most
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19 622 probable environmental and biological disease causing factors that induce complex biological
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21 623 network of events for disease pathogenesis and evolution; as well as on the protective and
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23 624 reparative mechanisms. We can additionally speculate that the nature of the intervention
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25 625 formula cannot be prohibitive for its use as preventive regimen and does not preclude
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27 626 probable positive efficacy on the other types of MS, but has to be further investigated. A
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29 627 larger size multicenter clinical trial will better establish PLP10 place in the armamentarium of
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31 628 treatments for MS.

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37 630 It is commonly accepted that nutrition is one of the possible environmental factors involved
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39 631 in the pathogenesis of MS, but its role as complementary MS treatment is unclear and largely
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41 632 disregarded.⁵⁷ It is well known that the majority of the patients suffering from MS they do
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43 633 use dietary supplements for a variable length of time and they prefer supplement type of
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45 634 “help” over conventional drugs.⁵⁸ Dietary antioxidants and fatty acids may influence the
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47 635 disease process in MS by reducing immune-mediated inflammation, oxidative stress and
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49 636 excitotoxic damage.¹¹ Present data reveal that healthy dietary molecules have a pleiotropic
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51 637 role and are able to change cell metabolism from anabolism to catabolism and down-regulate
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53 638 inflammation by interacting with enzymes, nuclear receptors and transcriptional factors.⁵⁷

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3 639 The present preliminary small size randomized controlled phase II clinical trial, for the first
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5 640 time provides link evidence between dietary, metabolic, immunological, and neurobiological
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7 641 aspects of MS after three quarters of a century of unsuccessful scientific efforts. This link
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9 642 evidence might probably be the beginning of opening new horizons and new avenues in the
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11 643 approach of MS prevention and treatment, and possibly of other multifactorial chronic
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13 644 diseases, including neurodegenerative and autoimmune as well.
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1.
Treatment Arms

A†	B (PLP10)†	C†	Placebo†
Intervention: EPA (1,650mg) / DHA (4,650mg) / GLA (2,000mg) / LA (3,850mg) / total other omega-3 (600mg)* / total MUFA** (1,714mg) + total SFA (18:0 160mg, 16:0 650mg) / vitamin A (0.6mg) / vitamin E (22mg) plus citrus aroma	Intervention: EPA (1,650mg) / DHA (4,650mg) / GLA (2,000mg) / LA (3,850mg) / total other omega-3 (600mg)* / total MUFA** (1,714mg) + total SFA (18:0 160mg, 16:0 650mg) / vitamin A (0.6mg) / vitamin E (22mg) + pure γ -tocopherol (760mg) plus citrus aroma	Intervention: pure γ -tocopherol (760mg) dispersed in pure virgin olive oil (16,137 mg) as delivery vehicle plus citrus aroma	Intervention: Olive oil (pure virgin) plus citrus aroma

* Other omega-3: C18:3n-3 37mg, C18:4n-3 73mg, C20:4n-3 98mg, C22:5n-3 392mg
 ** MUFA: 18:1 1300mg, 20:1 250mg, 22:1 82mg, 24:1 82mg
 † Total daily dose 19.5ml

EPAX1050, EPAX AS, Aalesund, Norway; was used as the source for the omega-3 PUFA, as re-esterified glycerides from fish body oils; Borage seed oil (organic, cold pressed) “Borago officinalis” Goerlich Pharma International GmbH, Edling, Germany, was used as the source for the omega-6 PUFA, MUFA and SFA, as triglycerides. The pure γ -tocopherol was purchased from Tama Biochemical Co. Ltd., Shinjuku-ku Tokyo, Japan; vitamin A as beta-carotene from HealthAid Ltd., Middlesex, United Kingdom and the Citrus aroma from Givaudan Schwaiz AG, Dubendorf, Switzerland.

Table 1. Intervention ingredients per treatment arm.

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2A.					
Characteristics	Group A (n=20)	Group B† (n=20)	Group C (n=20)	Placebo (n=20)	P- value
Sex					
Female - no. (%)	15 (75)	15 (75)	15 (75)	15 (75)	1.000
Age (yr)					
Mean ± Standard Deviation (SD)	38.0±11.9	36.9±8.4	37.7±8.7	38.1±10.9	0.982
Median (Range)	38.0 (22–65)	37.0 (25–61)	36.5 (24–54)	36.0 (21–58)	
Treatment history					
Patients on DMT- no. (%)	11 (55)	9 (45)	12 (60)	10 (50)	0.875
Pre-treatment disease duration (yr)					
Mean ± SD	9.0±7.6	8.6±4.8	8.6±5.3	7.7±5.7	0.909
Median (Range)	7.5 (2–37)	8.0 (2–20)	8.0 (3–24)	6.5 (2–25)	
Pre-treatment relapses‡					
Mean ± SD	2.33±1.68	2.41±1.73	2.31±1.66	2.10±1.32	0.946
Median (Range)	2.0 (1–6)	2.0 (1–7)	2.0 (1–6)	2.0 (1–4)	
ARR	1.17	1.21	1.16	1.05	0.946
Patients -% with ≤1 relapse	40	45	40	35	
Baseline EDSS score‡					
Mean ± SD	2.52±1.23	2.15±1.05	2.42±1.21	2.39± 0.93	0.775
Median (Range)	2.5 (1.0–5.5)	2.0 (1.0–4.0)	2.5 (0.0–5.0)	2.5 (1.0–4.0)	
2B.					
Characteristics	Group A (n=10)	Group B† (n=10)	Group C (n=9)	Placebo (n=12)	P- value
Sex					
Female - no. (%)	5 (50)	7 (70)	6 (66.6)	10 (83.3)	0.419
Age (yr)					
Mean ± SD	36.6±13.5	34.80±5.4	40.9±8.1	39.8±13.2	0.572
Median (Range)	34.5 (22–65)	34.5 (26–43)	40.0 (29–54)	37.5 (21–58)	
Treatment history					
Patients on DMT- no. (%)	6 (60)	4 (40)	6 (67)	6 (50)	0.949
Pre-treatment disease duration (yr)					
Mean ± SD	9.7±10.0	8.3±5.3	11.3±6.1	8.7± 7.1	0.807
Median (Range)	7.5 (2–37)	8.0 (2–20)	8.0 (4–24)	5.5 (2–25)	
Pre-treatment relapses					

Mean ± SD	2.20±1.47	2.70±1.25	1.78±0.66	1.67±1.37	0.241
Median (Range)	2.0 (1 – 6)	2.5 (1 – 4)	2.0 (1 – 3)	1.5 (1 – 4)	
ARR	1.10	1.35	0.89	0.83	
Patients -% with ≤1 relapse	30	20	33	50	
Baseline EDSS score					
Mean ± SD	2.65±1.37	2.40±1.12	2.11±1.02	2.16±0.96	0.698
Median (Range)	3.0 (1.0–5.5)	2.5 (1.0–4.0)	2.0 (1.0–4.0)	2.0 (1.0–3.5)	

† PLP10 group

‡ Available data at Entry Baseline (n=18 for group A, n=17 for group B, n=19 for group C, n=19 for group D)

Table 2. The table section 2A reports the demographics and baseline disease characteristics for total randomized population by treatment arm.

The table section 2B reports the demographics and baseline disease characteristics of all-time on-study population by treatment arm. There were no significant between study-group differences at baseline for any characteristic.

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3A.								
Characteristics	Group A (N=10)		Group B† (N=10)		Group C (N=9)		Placebo (N=12)	
End Point	X	Y	X	Y	X	Y	X	Y
Total No. of relapses	22	17	27	8	16	13	20	25
Annual Relapse Rate (ARR)	1.10	0.85	1.35	0.40	0.88	0.72	0.83	1.04
% Reduction Compared to Placebo (primary endpoint) (Ys)¶	-18		-62		-30		N/A	
P Value against placebo	0.468		0.024		0.578			
ARR change -% (Y to X)¶	-23		-70		-18		+25	
P value against baseline	0.425		0.003		0.578		0.500	
X: Total number of relapses of 24 months pre-treatment (baseline) Y: Total number of relapses of 24 months on-treatment ¶ Unadjusted estimate								
3B.								
Excluding patients on natalizumab	Group A (N=9)		Group B† (N=10)		Group C (N=9)		Placebo (N=10)	
End Point	X	Y	X	Y	X	Y	X	Y
Total No. of relapses	16	15	27	8	16	13	13	19
Annual Relapse Rate (ARR)	0.88	0.83	1.35	0.40	0.88	0.72	0.65	0.95
% Reduction Compared to Placebo (primary endpoint) (Ys)¶	-13		-58		-24		N/A	
P Value against placebo	0.493		0.016		0.412			
ARR change -% (Y to X)¶	-6		-70		-18		+46	
P value against baseline	0.857		0.003		0.578		0.354	
X: Total number of relapses of 24 months pre-treatment (baseline) Y: Total number of relapses of 24 months on-treatment † PLP10 group ¶ Unadjusted estimate								
Table 3. The table section 3A reports the two year primary end points of ARR of all-time on-study population by treatment arm and percent difference with placebo. During the 24mo period on-treatment								

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3 the ARR of the group A was 0.85 with 18% decrease compared to placebo (p=0.468), of PLP10 group
4 0.40 with 62% decrease (p=0.024), and of group C 0.72 with 30% decrease (p=0.578) and reports the
5 comparison of the 24mo pre-treatment ARR (baseline ARR) to 24mo on-treatment ARR of all-time on-
6 study population including patients on natalizumab.
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14 The table section 3B reports comparison of the 24mo pre-treatment ARR to 24mo on-treatment ARR of
15 all-time on-study population excluding patients on natalizumab; and the comparison of the ARR during
16 the 24mo period on-treatment (primary end point) between each one of the groups against placebo.
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4A.									
Characteristics	Group A (N=8)		Group B† (N=7)		Group C (N=10)		Placebo (N=7)		
	X	Y	X	Y	X	Y	X	Y	
No. of Relapses	20	14	14	14	27	26	20	13	
Annual Relapse Rate (ARR)	1.25	0.88	1.00	1.00	1.35	1.30	1.42	0.92	
X: Total number of relapses of 24 months pre-treatment Y: Total number of relapses of 24 months on-treatment									
4B.									
Characteristics	Group A (N=20)		Group B† (N=20)		Group C (N=20)		Placebo (N=20)		
End Point	X	Y	X	Y	X	Y	X	Y	
No. of Relapses	45	34	49	30	46	41	43	41	
Annual Relapse Rate (ARR)	1.13	0.85	1.23	0.75	1.15	1.03	1.08	1.03	
ARR Reduction -% (Y to X)¶	-25		-39		-10		-5		
P value against baseline	0.120		0.005		0.475		0.652		
% Reduction of the ARR Compared to Placebo (Ys)¶	-18		-27		0.0		N/A		
P Value against placebo	0.447		0.121		0.996				
X: Total number of relapses of 24 months pre-treatment (baseline) Y: Total number of relapses of 24 months on-treatment ¶ Unadjusted estimate † PLP10 group									

Table 4. The table section 4A reports the two year primary end point of relapses based on study design as reported by drop-out patients by Treatment Arm. The most drop-out patients that went on disease modified therapy (DMT) were from the group A and placebo with three and two patients respectively on natalizumab. These parameters justify the decreased number of relapses recorded within group A and placebo drop-outs; and this could affect the ITT analysis in favor of placebo when the total two-year recorded data are used. For PLP10 group, 14 relapses were reported at baseline and remained the same during the two-year study. For placebo, 20 relapses were reported at baseline and decreased to 13 during the two-

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3 year study. This is expected since, for PLP10 group 43% of the drop-outs were under DMT at
4 entry baseline and remained the same until the end of the study with no patient on
5 natalizumab; but 57% of the placebo group drop-outs that were under DMT at entry baseline
6 increased to 86% at the end of the study including two patients on natalizumab.
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14 The table section 4B reports comparison of 24 months pre-treatment ARR (baseline) to 24
15 months on-treatment ARR of total randomized population, by treatment arm. The ARR of
16 PLP10 group was 1.23 at baseline and 0.75 at the end of the study (39% reduction $p=0.005$),
17 and for placebo 1.08 at baseline and 1.03 at the end of the study (5% reduction $p=0.652$). No
18 statistical difference was calculated for the other two treatment arms. During the 24 months
19 on-treatment, PLP10 group presented a 27% reduction of ARR versus placebo ($p=0.121$),
20 with all groups without statistically significant results.
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5.					
Characteristics*	Group A (n=10)	Group B PLP10 (n=10)	Group C (n=9)	Placebo (n=12)	P-value Group B vs. Placebo
Annual relapse rate over 1 year**	0.80	0.40	0.78	0.83	
Total number of relapses**	8	4	7	10	
Primary end points					
Annual relapse rate over 2 years (95% CI)**	0.85	0.40 (0.15-0.87)	0.72	1.04	0.024
Total number of relapses**	17	8	13	25	
Excluding patients on natalizumab					
Annual relapse rate over 2 years (95% CI)	0.83	0.40 (0.10-0.79)	0.72	0.95	0.016
Total number of relapses	15	8	13	19	
Secondary end points					
Cumulative probability of sustained progression increase by 1 point on EDSS confirmed after 6 mo, over 2 years -% **	43	10 (1/10)	24	58 (7/12)	0.019
Excluding patients on natalizumab					
cumulative probability of sustained progression increase by 1 point on EDSS confirmed after 6 mo, over 2 years -%	33	10 (1/10)	24	70 (7/10)	0.006
Exploratory Results					
Patients proportion with ≤1 relapse over 2 years -% **	50 (5/10)	90 (9/10)	56 (5/9)	42 (5/12)	0.030
MRI					
Patients proportion with new or enlarging T2 lesions-% **	----	29 (2/7)	----	67 (4/6)	
Excluding patients on natalizumab					
Patients proportion with no new or enlarging T2 lesions-%	----	29 (2/7)	----	80 (4/5)	
DMT (interferons, glatiramer acetate) and natalizumab					
Patients proportion on DMT and natalizumab at the end of 2 years-% **	80 (8/10)†	60 (6/10)	67 (6/9)	75 (9/12) ‡	0.747
* CI denotes confidence interval.					
** Including patients on natalizumab					
† 1 out of 10 on natalizumab					
‡ 2 out of 12 on natalizumab					

Table 5. Clinical end points, according to study group for all-time on-study population.

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32 730 authors critically revised and approved the final version. M.C.P and I.S.P were responsible
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34 731 for the protocol and study design. M.C.P was the evaluator of patients' clinical progress signs
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27 751 No pharmaceutical companies were involved in this phase II clinical trial. The intervention is
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29 752 under a USA provisional patent; Application Number 61469081.
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34 754 Ethical approval: Cyprus National bioethics committee (reference EEBK/EII/2005/10).
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38 756 All authors have completed the Unified Competing Interest form at
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Article Summary

Article focus:

- The increasing prevalence of Multiple Sclerosis (MS), the limited efficacy and the side effects of the existing treatments urge the clinical need for the development of new, innovative, more effective, safe, and preventive treatment strategies.
 - For the first time we propose three original nutraceutical treatment intervention-cocktails, formulated based on systems medicine through nutritional systems biology rational; with PLP10 representing the complete composition of the formulation and the other two treatments represent partial formulations.
 - We have studied the proposed interventions on 80 relapsing remitting MS patients (four groups of 20 each) for a total of 42 months, in a randomized, double-blind, placebo-controlled, phase II proof-of-concept clinical trial.
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Key messages:

- The per-protocol data analyses indicated that PLP10 is associated with significant efficacy versus placebo on both reducing the annualized relapse rate and disease progression without any adverse or severe side effects.
- Overall, for this small size phase II study, a total of 41 (51%) patients completed the 42-month trial. For the per-protocol analysis of the two-year primary end point, 8 relapses were recorded in the PLP10 group (n=10) (0.40 ARR) versus 25 relapses in the placebo group (n=12) (1.04 ARR), a 64% adjusted relative rate reduction for the PLP10 group (RRR 0.36, 95% CI 0.15 to 0.87, p=0.024). In a subgroup analysis that excluded patients on monoclonal antibody (natalizumab) treatment, the observed adjusted RRR became stronger (72%) over the two years (RRR 0.28, 95% CI 0.10 to 0.79, p=0.016). Per-protocol analysis for the secondary outcome at two years, time to disability progression, was significantly longer only with PLP10. The cumulative probability of disability progression at two years was 10% in the PLP10 group and 58% in the placebo group (unadjusted log-rank p=0.019). In a subgroup analysis that excluded patients on natalizumab the cumulative probability of progression was 10% for the 10 patients in the PLP10 group and 70% for the 12 patients in the placebo group, a relative 86% decrease in the risk of sustained progression of disability in the PLP10 group (unadjusted log-rank p=0.006; adjusted hazard ratio, 0.11; 95% CI 0.01 to 0.97, p=0.047).
- PLP10 has probably the ability to interfere with the total known repertoire of mechanisms involved in MS pathogenesis as well as with the recovery mechanisms, neuroprotection and remyelination.; it is potentially able to manipulate global perturbed networks of the disease causing factors rather than focusing only on unique failing components.
- This proof-of-concept clinical study might be indicative and the beginning of new avenues in MS prevention and treatment and of new epoch of novel drug development

with dynamic therapeutic potential for chronic complex multifactorial diseases.

Strengths and limitations of this study:

- The strength of this study is the systems medicine therapeutic approach, the length of the study the inclusion of the six months normalization (chronotherapy) period, and the study protocol following all indicated appropriate guidelines with definite inclusion/exclusion criteria and primary/secondary end points.
- The sample size and the high rate of drop-outs (palatability of the formula) are the limitations associated with the present study.
- A phase III multi-center clinical trial will establish the present intervention regime among the best choices among neurodegenerative diseases prophylaxis and MS treatment drugs faretra.

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3 927 **Figure legends**
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6 928 **Figure 1.** Study Flowchart
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9 929 **Figure 2.** Omega-6 and omega-3 PUFA, their respective metabolic derivatives and their
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11 possible effects on inflammation.
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15 931 After consumption, the PUFAs are metabolized via several pathways (not shown) to active
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17 932 compounds that mediate inflammation and products that promote resolution of inflammation.
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20 933 Abbreviations: PL, phospholipid; IFN- γ , interferon γ ; IL-2, interleukin 2; NF κ B, nuclear
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22 934 factor kappa B; PGE₂, prostaglandin E₂; PPAR γ , peroxisome proliferator-activated receptor
23
24 935 γ ; PUFAs, polyunsaturated fatty acids; TGF β , transforming growth factor β ; TNF, tumor
25
26 936 necrosis factor; COX, cyclooxygenase; hydroxyeicosatetraenoic acid; HPETE,
27
28 937 hydroperoxyeicosatetraenoic acid; LOX, lipoxygenase; LT, leukotriene; PG, prostaglandin;
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30 938 TX, thromboxane; RXR- γ , retinoid X receptor-gamma; Nrf2, nuclear respiratory factor;
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32 939 MMP, metalloproteinase.
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37 940 **Figure 3.** Panel A demonstrates the ARR of all-time on-study patients during the 24mo pre-
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39 941 treatment (baseline ARR) and at different on-study intervals (6, 12, 18, 24 mo) per treatment
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41 942 arm. **
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44 943 Panel B demonstrates the ARR of all-time on-study population between 0-6, 6-12, 6-18, and
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46 944 6-24 mo period intervals, of PLP10 vs. placebo group. **
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48
49 945 Panel C demonstrates the ARR of all-time on-study population of PLP10 vs. placebo group at
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51 946 baseline, during 1st year, and during the 2-year on-treatment. **
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55 947 Panel D demonstrates the dispersion of relapses throughout the 2-year period of all-time on-
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57 948 study (excluding patients on natalizumab) of PLP10 (n=10) vs. placebo (n=10). Placebo
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3 949 shows an irregular dispersion of relapses in relation to PLP10 group with linear increasing
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5 950 trend while PLP10 shows a stabilized linear trend. By using the per-protocol model where
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7 951 patients on natalizumab were excluded, we could compare the number of relapses on a same
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9 952 number of patients.

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13 953 ** Including the patients on natalizumab.

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16 954 **Figure 4.** Panel 4A demonstrates the Kaplan–Meier plot of the time to sustained progression
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18 955 of disability among all-time on-study patients, excluding patients on natalizumab, receiving
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20 956 intervention A, PLP10 and C as compared with placebo. PLP10 reduced the risk of sustained
21
22 957 progression of disability by 86% over two years (p=0.006). Intervention formula A reduced
23
24 958 the risk of sustained progression of disability by 53% (p=0.266) and intervention formula C
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26 959 by 67% (p=0.061).

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30 960 Panel 4B demonstrates the Kaplan–Meier plot of the time to sustained progression of
31
32 961 disability among ITT population receiving intervention A, PLP10 and C as compared with
33
34 962 placebo. PLP10 reduced the risk of sustained progression of disability by 71% over two years
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36 963 (p=0.052, trend). Intervention formula A reduced the risk of sustained progression of
37
38 964 disability by 22% (p=0.727) and intervention formula C by 40% (p=0.447).

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41 965 **Figure 5.** Mean change in expanded disability status scale score as a function of visit
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43 966 number. Values are expressed as mean ± s.e.m.

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47 967 ¶ Including patients on natalizumab

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50 968 ¶¶ Excluding patients on natalizumab

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1 **A novel oral nutraceutical formula of omega-3 and omega-**
2 **6 fatty acids with vitamins (PLP10) in relapsing remitting**
3 **multiple sclerosis: a randomized, double-blind, placebo-**
4 **controlled proof-of-concept clinical trial**

5 **Marios C. Pantzaris***, George N. Loukaides, Evangelia E. Ntzani, Ioannis
6 **S. Patrikios***

7 * Both M.C.P and I.S.P are the first authors and both are the corresponding authors

8
9 The Cyprus Institute of Neurology and Genetics (CING) 1683 Nicosia, Cyprus Marios C.
10 Pantzaris Senior Consultant Neurologist Neurology Clinic C and PALUPA Medical Ltd., The
11 Cyprus Institute of Neurology and Genetics (CING) 1683 Nicosia, Cyprus George N.
12 Loukaides Clinical Dietitian/Nutritionist Neurology Clinic and PALUPA Medical Ltd.,
13 University of Ioannina School of Medicine (UISM) 45110 Ioannina, Greece Evangelia E.
14 Ntzani Assistant Professor Clinical and Molecular Epidemiology Unit, Department of
15 Hygiene and Epidemiology, PALUPA Medical Ltd at The Cyprus Institute of Neurology and
16 Genetics (CING) 1683 Nicosia, Cyprus Ioannis S. Patrikios Senior Research Scientist,
17 visiting Professor at European University Cyprus Nicosia, Cyprus, Department of Health and
18 Science. Correspondence should be addresses to e-mail: I.Patrikios@euc.ac.cy or
19 pantzari@cing.ac.cy

1
2
3 22 **Correspondence to:**
4

5 23 **Ioannis Patrikios**

6
7 24 The Cyprus Institute of Neurology and Genetics (CING)

8
9 25 Neurology Clinic C (PALUPA Medical),

10
11 26 6 International Airport Av.

12
13 27 P.O.Box 23462, 1683 Ayios Dometios. Nicosia, Cyprus

14
15 28 Tel: +357 22 358 600, +357 99 097 856;

16
17 29 i.patrikios@euc.ac.cy

18
19 30 patrikiosioannis@gmail.com
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32 **AND**

33
34 **Marios Pantzaris**

35 The Cyprus Institute of Neurology and Genetics (CING)

36 Neurology Clinic C (PALUPA Medical),

37 6 International Airport Av.

38 P.O.Box 23462, 1683 Ayios Dometios, Nicosia Cyprus

39 Tel: +357 22 358 600; +357 99 677 067

40 pantzari@cing.ac.cy
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42 **Keywords:** antioxidant vitamins, polyunsaturated fatty acids, multiple sclerosis, systems
43 medicine, randomized clinical trial.
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56 **Word Count:** 6299
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1
2
3 **Abstract**
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5 **Objective** To assess whether our three novel interventions, formulated based on systems
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medicine therapeutic concept reduce disease activity in patients with relapsing remitting multiple sclerosis who were either treated with disease modifying treatment or untreated.

Design 30-month randomized double-blind, placebo-controlled, parallel design, phase II proof-of-concept clinical study.

Settings Cyprus Institute of Neurology and Genetics (CING)

Participants and Interventions 80 subjects were randomized into four groups of 20. The first intervention (A) was composed of omega-3 and omega-6 polyunsaturated fatty acids at 1:1 wt/wt. Specifically, the omega-3 fatty acids were docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) at 3:1 wt/wt and the omega-6 fatty acids were linoleic acid (LA) and gamma (γ)-linolenic acid (GLA) at 2:1 wt/wt. This intervention also included minor quantities of other specific polyunsaturated, monounsaturated and specific saturated fatty acids as well as vitamin A and vitamin E (alpha-tocopherol). The third intervention (C) was γ -tocopherol alone. The second intervention (PLP10) was a combination of A and C. A fourth group of 20 received a vehicle placebo. The interventions were administered per os once daily.

Main outcome measures The primary endpoint was the annualized relapse rate (ARR) of the three interventions versus placebo at two years. The secondary end point was the time to confirmed disability progression at two years.

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3 71 **Results** A total of 41 (51%) patients completed the 42-month trial. Overall, for the per-
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5 72 protocol analysis of the two-year primary end point, 8 relapses were recorded in the PLP10
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7 73 group (n=10) (0.40 ARR) versus 25 relapses in the placebo group (n=12) (1.04 ARR), a 64%
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9 74 adjusted relative rate reduction for the PLP10 group (RRR 0.36, 95% CI 0.15 to 0.87,
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11 75 p=0.024). In a subgroup analysis that excluded patients on monoclonal antibody
12
13 76 (natalizumab) treatment, the observed adjusted RRR became stronger (72%) over the two
14
15 77 years (RRR 0.28, 95% CI 0.10 to 0.79, p=0.016). Per-protocol analysis for the secondary
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17 78 outcome at two years, time to disability progression, was significantly longer only with
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19 79 PLP10. The cumulative probability of disability progression at two years was 10% in the
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21 80 PLP10 group and 58% in the placebo group (unadjusted log-rank p=0.019). In a subgroup
22
23 81 analysis that excluded patients on natalizumab the cumulative probability of progression was
24
25 82 10% for the 10 patients in the PLP10 group and 70% for the 12 patients in the placebo group,
26
27 83 a relative 86% decrease in the risk of sustained progression of disability in the PLP10 group
28
29 84 (unadjusted log-rank p=0.006; adjusted hazard ratio, 0.11; 95% CI 0.01 to 0.97, p=0.047). No
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31 85 adverse events were reported. Interventions A (10 patients) and C (9 patients) showed no
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33 86 significant efficacy.
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41 88 **Conclusions** In this small proof-of-concept randomized double-blind clinical trial, PLP10
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43 89 treatment significantly reduced the ARR, and the risk of sustained disability progression
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45 90 without any reported serious adverse events. Larger studies are needed to further assess the
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47 91 safety and efficacy of PLP10.
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52 93 **Trial registration** International Standard Randomized Controlled Trial, number
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54 94 ISRCTN87818535.
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96 Introduction

97 Multiple sclerosis (MS) is a complex multifactorial disease that results from interplay
98 between as yet unidentified environmental factors and susceptibility genes.¹⁻³ Together, these
99 factors trigger a cascade of events, involving engagement of the immune system,
100 inflammatory injury of myelin, axons and glia, functional recovery and structural repair,
101 gliosis, and neurodegeneration.⁴ The bio-mechanisms involved are: immune-mediated
102 inflammation, oxidative stress and excitotoxicity.⁵⁻⁹ These mechanisms may all contribute to
103 oligodendrocyte and neuronal damage and even cell death, hence promoting disease
104 progression. The increasing prevalence of MS, the limited efficacy and the side effects of the
105 existing treatments urge the clinical need for the development of new, innovative, more
106 effective, safe, and preventive treatment strategies.

107
108 Research has shown that multiple variables dynamically interact and many different complex
109 interrelated processes are simultaneously orchestrated for MS pathogenesis. The fundamental
110 distinctiveness of systems medicine (SM) is not just the recognition that different specific
111 complex factors are important in disease management, but that they need to be incorporated
112 in some meaningful way to treatment selection and delivery.¹⁰ The primary challenge tackled
113 by systems scientific approach is the elucidation of how these multiple variables dynamically
114 interact and how one can apply this understanding to affect the system and achieve a
115 desirable end.¹⁰ The answer might be the simultaneous interference with all involved
116 perturbed mechanisms, by using a cocktail of different specific ingredients, potentially able
117 through synergistic effect to give a long, holistic and effective treatment (Supplementary
118 Information Methods 1).

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3 120 The polyunsaturated fatty acids (PUFA) composition of membrane phospholipids plays a
4
5 121 direct role in immune and non-immune related inflammation. PUFA and antioxidant
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7 122 deficiencies along with decreased cellular antioxidant defense mechanisms have been
8
9 123 reported for MS patients.¹¹The cause of these PUFA deficiencies is not entirely clear and may
10
11 124 involve metabolic and nutritional alterations.¹¹
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16 126 Increased or uncontrolled inflammation contributes to several different acute and chronic
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18 127 diseases and it is characterized by the production of inflammatory cytokines, arachidonic acid
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20 128 (AA)-derived eicosanoids (prostaglandins (PGs), thromboxanes (TXs), leukotrienes (LTs),
21
22 129 and other oxidized derivatives), other inflammatory agents such as reactive oxygen species
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24 130 (ROS) , nitric oxide (NO), and adhesion molecules (Fig 2).¹² During inflammation glutamate
25
26 131 homeostasis is altered by activated immune cells releasing increased quantities of glutamate
27
28 132 that can result in over activation of glutamate receptors and in return excitotoxic
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30 133 oligodendroglial death.^{7, 13} As such, among others, membrane-related pathology, immune-
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32 134 mediated inflammation, oxidative stress and excitotoxicity provide potentially useful
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34 135 combined targets for intervention in MS.
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41 137 *In vitro and in vivo* studies have demonstrated that dietary EPA, DHA, LA, and GLA can be
42
43 138 implicated and modulate almost all known complex network of events and pathways
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45 139 repertoire in MS pathophysiology. Brain membrane fatty acid composition can be modified
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47 140 with dietary supplementation, but the process has been showed to be age dependent (it takes
48
49 141 much longer in adults versus developing brains) as well as possibly dependent on the
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51 142 quantities of the dietary/supplemented PUFAs.¹⁴ Both human and animal studies proved that
52
53 143 diets high in DHA and EPA increase the proportion of these PUFA in the membranes of
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55 144 inflammatory cells and reduce the levels of AA.^{12, 15} The anti-inflammatory properties of
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3 145 omega-3 include production of PGs and TXs of the 3-series and LTs of the 5-series (Fig 2).¹⁴
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5 146 ¹⁶ Resolvins and protectins are biosynthesized from omega-3 fatty acids via cyclooxygenase-
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7 147 2/lipoxygenase (COX-2/LOX) pathways and they promote control of inflammation in neural
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9 148 tissues (Fig 2).¹⁷⁻²¹ T-cell proliferation in acute and chronic inflammation can be reduced by
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11 149 supplementation with either omega-6 or omega-3 PUFA.²² Furthermore, vitamin E is an
12
13 150 important antioxidant that can interrupt the propagation of free radical chain reactions.²³
14
15 151 Vitamin E (alpha-tocopherol, an isoform of vitamin E) efficiently detoxifies hydroxyl,
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17 152 perhydroxyl and superoxide free radicals.²⁴ However γ -tocopherol (another isoform of
18
19 153 vitamin E) seems to be more efficiently implicated in trapping NO radicals.²⁵ In addition
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21 154 alpha-tocopherol exerts non-antioxidant properties, including modulation of cell signaling
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23 155 and immune function, regulation of transcription, and induction of apoptosis.²⁶
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29 157 Moreover, omega-3 fatty acids electrophilic derivatives formed by COX-2, in activated
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31 158 macrophages can stimulate the nuclear respiratory factor (Nrf2) inducing the transcription of
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33 159 neuroprotective and antioxidant related genes, and can activate the peroxisome proliferator-
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35 160 activated receptor (PPAR) γ for anti-inflammatory response.²⁷⁻²⁹ In animal studies, EPA and
36
37 161 DHA proved to be endogenous ligands of RXRs, with positive effect on neurogenesis.³⁰
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39 162 Additionally, in 2008 Salvati and coworkers reported evidense of accelerated myelination in
40
41 163 DHA- and EPA-treated animals.³² Moreover, DHA and EPA are reported to significantly
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43 164 decrease the levels of metalloproteinases (MMP) -2, -3, -9, and -13 with a significant role in
44
45 165 the migration of lymphocytes into the CNS by inducing disruption of the blood brain barrier
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47 166 (BBB), an important step in the formation of MS lesions.³³⁻³⁹
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53 168 Based on the above, specific PUFA and antioxidant vitamins fulfill the criterion of biologic
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55 169 plausibility and have the potential to diminish MS symptoms severity and activity, even
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3 170 promoting recovery (remyelination).¹¹ Overall, PLP10 contains multiple ingredients (omega-
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5 171 3, omega-6 and other fatty acids and vitamins) potentially able to modulate key
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7 172 interconnected components (i.e. genes, proteins) and structural molecules (i.e. cellular
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9 173 membrane lipids, receptors) within the functional network of events of MS pathogenesis.⁴⁰
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14 175 This is a randomized phase II, single-center, double-blind, placebo-controlled, proof-of-
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16 176 concept clinical trial evaluating the therapeutic ability of PLP10 and of two other
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18 177 interventions (A and C) consisting of PLP10 constituent partial fractions (Table 1) versus
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20 178 placebo on RRMS patients.
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28 181 **Methods**

29 182 **Patients**

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32 183 Eligibility criteria were an age of 18 to 65; a diagnosis of RRMS according to the McDonald
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34 184 criteria; a score of 0.0 to 5.5 on the EDSS, a rating that ranges from 0 to 10, with higher
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36 185 scores indicating more severe disability; MRI showing lesions consistent with MS; and at
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38 186 least one documented clinical relapse either receiving or not disease modifying treatment
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40 187 (DMT) within the 24 months period before beginning (enrollment) of the study. Patients were
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42 188 excluded because of a recent (<30 days) relapse, prior immunosuppressant or monoclonal
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44 189 antibodies therapy, pregnancy or nursing, other severe disease compromising organ function,
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46 190 progressive MS, history of recent drug or alcohol abuse, use of any additional food
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48 191 supplement, vitamin, or any form of PUFA, history of severe allergic or anaphylactic
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50 192 reactions or known specific nutritional hypersensitivity. No monitor or limitations on
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52 193 patients' daily diet habits were included in the study design since the quantities of the
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54 194 ingredients within the formulas daily-dosage could not be significantly affected or spoiled by
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3 195 any confounding factors within any known global daily food diet (see procedures, treatment
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5 196 regimen and end-points).

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9 198 The study was conducted in accordance with the standards of the International Conference of
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11 199 Harmonization Guidelines for Good Clinical Practice. The protocol was developed by the
12
13 200 investigators and it was approved by the Cyprus National Bioethics Committee and was
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15 201 overseen by an independent safety-monitoring committee evaluating the safety and over-all
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17 202 benefit-risk profiles. The adherence of care providers with the protocol was assessed by an
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19 203 external committee assigned by the funder of the project through reviews of case report
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21 204 forms. All patients gave written informed consent at the time of enrolment.
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26 27 206 **Randomization and masking**

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29 207 Patients were randomly assigned to four intervention groups in a 1:1:1:1 ratio stratified by
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31 208 gender (women to men, 3:1). Randomization was facilitated by a lottery-type pool of
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33 209 numbered balls. Patients were randomly assigned to treatment in blocks of four by flipping a
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35 210 coin as follows: for the first two drawn balls heads stratified them to the groups A/B and tails
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37 211 stratified them to the groups C/D. The other two balls were stratified accordingly. A second
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39 212 toss of the coin assigned the two patients to group A (head)/B (tail) or group C (head)/D
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41 213 (tail). The randomization scheme was generated, performed and securely stored by Helix
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43 214 Incubator Organization of Nicosia University (HIONU).
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49 216 The interventions had identical appearance and smell in dark bottles (15 daily-dose
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51 217 portions/bottle) under nitrogen bed and labeled by HIONU with code numbers, unidentifiable
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53 218 for both patients and investigators. Study data were collected by the investigators and saved
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55 219 by the HIONU that also held the blinded codes of the study. All study personnel involved in
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3 220 the conduct of the study were blinded throughout the study. Treating/examining physician,
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5 221 other investigators, pharmacist, neuroradiologist and patients were masked to treatment
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7 222 allocation.
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11 224 **Procedures and end points**

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14 225 The specific omega-3 (re-esterified glycerides) and omega-6 (glycerides) raw materials were
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16 226 purchased according to the required interventions' PUFA-fraction specification (molecular
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18 227 structure, quantity/ratio and quality) with vitamin E (alpha-tocopherol) used as antioxidant
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20 228 stabilizer by the supplier. The vitamins and masking aroma were purchased separately. The
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22 229 mixing of fractions to the final required intervention-composition specification was always
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24 230 performed by the same team of scientists under the supervision of the involved medical
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26 231 biochemist and lipidology specialist, under appropriate conditions every six months.
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28 232 Interventions were stored refrigerated in dark until use. See Table 1 and Supplementary
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30 233 Information Methods 1 and 2 for intervention specification detailed description and
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32 234 study/intervention rational.
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38 236 Participants were randomly assigned to receive: in group A, a daily dose of a 19.5ml
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40 237 mixture of EPA (1,650mg) / DHA (4,650mg) / GLA (2,000mg) / LA (3,850mg) / total other
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42 238 omega-3 (600mg) / total MUFA (1,714mg) + total SFA (18:0 160mg, 16:0 650mg) / vitamin
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44 239 A (0.6mg) / vitamin E (22mg) plus citrus-aroma (intervention A); in group B PLP10, a daily
45
46 240 dose of a 19.5ml mixture of EPA (1,650mg) / DHA (4,650mg) / GLA (2,000mg) / LA
47
48 241 (3,850mg) / total other omega-3 (600mg) / total MUFA (1,714mg) + total SFA (18:0 160mg,
49
50 242 16:0 650mg) / vitamin A (0.6mg) / vitamin E (22mg) plus pure γ -tocopherol (760mg) plus
51
52 243 citrus-aroma (intervention B); in group C, a daily dose of a 19.5ml mixture of pure γ -
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54 244 tocopherol (760mg) dispersed in pure virgin olive oil (16,137 mg) plus citrus-aroma
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3 245 (intervention C) and in group D placebo, a daily dose of a 19.5ml mixture of pure virgin
4
5 246 olive oil (16,930mg) plus citrus-aroma (intervention D) (Table 1). The institution's
6
7 247 pharmacist was responsible for the appropriate storage and handling of the interventions to
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9
10 248 the individual participants. The interventions were taken orally once daily 30 minutes before
11
12 249 dinner by a dosage calibrated cup for 30 months. The ingredients, ratio and dose have been
13
14 250 selected based on their biophysical interrelation to the total known multiple MS causing
15
16 251 factors, their biochemical importance and the role expected to play in the normalisation and
17
18 252 treatment of the involved complex network of events in the disease pathophysiology.
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21 253 Moreover, the high intake dosage was used to overcome any abnormal dietary accumulation
22
23 254 of related agents as a result of patients' food intake habits, irrespective of geographical origin,
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25 255 in relation to the daily consumption ratio of the total fatty acid intake; in order to end-up with
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27 256 omega-3 to omega-6 PUFA indicated physiological body ratio composition of 1:1 wt/wt.
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32 258 The period beginning from July 1st 2007 (enrolment) until December 31st 2007 (entry
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34 259 baseline) was used for normalization period. This six-month normalization period would
35
36 260 allow the interventions' agents to exert their beneficial effect (for the
37
38 261 incorporation/normalization of cell membranes by oral PUFA, since they need four to six
39
40 262 months to exert pivotal action on immune and neural cells, correction of antioxidant
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42 263 deficiency and body PUFA, and optimal normalization of EPA and DHA levels/ratio).⁴¹⁻⁴³
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44
45 264 The study was completed on December 31st 2009 and the recording of relapses continued
46
47 265 until December 31st 2010. More clearly the study included the "normalization period" (July
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49 266 1st 2007 to Dec 31st 2007), the "on treatment" period (Jan 1st 2008 to Dec 31st 2009) and the
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51 267 12-month "extended period" (Jan 1st 2010– Dec 31st 2010).
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3 269 Depending on their clinical status and in accordance with the ethical issues governing clinical
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5 270 trials participants continued receiving the indicative regular available treatments, according to
6
7 271 international guidelines with persistent evaluation of any side-effects and adverse events.
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9
10 272 The study was designed to end 30 months after enrolment and clinical assessments were
11
12 273 scheduled at entry baseline, 3, 9, 15, 21 and 24 months on-treatment. Patients were also
13
14 274 clinically examined by the treating neurologist within 48 hours after the onset of new or
15
16 275 recurrent neurologic symptoms.
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21 277 The primary end point was the ARR at two years. A relapse was defined as new or recurrent
22
23 278 neurologic symptoms not associated with fever or infection that lasted for at least 24 hours
24
25 279 and accompanied by new neurologic signs. Relapses were treated with methyl-prednisolone
26
27 280 at a dose of 1g intravenous per day, for three days followed by prednisone orally at a dose of
28
29 281 1mg/kg of weight per day on a tapering scheme for three weeks. The secondary end point at
30
31 282 two years was the time to confirmed disability progression, defined as an increase of 1.0 or
32
33 283 more on EDSS, confirmed after six months (progression could not be confirmed during a
34
35 284 relapse). The final EDSS score was confirmed six months after the end of the study. A post-
36
37 285 hoc analysis was performed assessing the proportion of patients free from new or enlarging
38
39 286 T2 lesions on brain MRI scans at the end of the study for the per-protocol participants of the
40
41 287 group receiving the highest effective intervention versus placebo. Comparison was made only
42
43 288 versus the available archival MRI scans up to three months before the enrolment date. MRI
44
45 289 scans were performed and blindly analyzed at an MRI evaluation centre. The patients
46
47 290 continued to be followed for additional 12 months after completion of the trial and relapses
48
49 291 were recorded. Finally, patients were strongly encouraged to remain in the study for follow-
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51 292 up assessments even if they had discontinued the study drug.
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3 294 Blood samples were collected from all randomized patients at the time of enrolment, at every
4
5 295 scheduled clinical assessment and during relapses. To check individual compliance with
6
7 296 intake, the fatty acids composition of patients' red blood cells' membranes was determined,
8
9 297 by gas chromatography, according to a standard protocol. The fatty acid analyses were
10
11 298 performed after study termination and thus did not influence the blinding.
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15
16 300 The involved neurologist was experienced with more than 20 years in practice and trained to
17
18 301 standardise EDSS scoring procedures, examined patients, made all medical decisions,
19
20 302 determined the EDSS score and reviewed adverse or side-effects. The medical biochemist,
21
22 303 specialist on lipidology and immunology and the registered clinical dietitian, members of the
23
24 304 investigator team were experienced with more than 25 years in practice. Patients were able to
25
26 305 contact the neurologist at any time if there was any adverse event, side-effect or allergic
27
28 306 reaction. The study drug was not suspected to have any clinical or laboratory adverse effects
29
30 307 different from placebo that could disturb the double-blind nature of the trial. Therefore, the
31
32 308 same study-neurologist functioned as both the treating and evaluating physician.
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38 310 Safety measures were assessed from the time of enrollment until 12 months following study
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40 311 completion. Haematological and biochemical tests were performed at enrolment and at every
41
42 312 12 months, including full blood count, renal and liver function tests, proteins, cholesterol,
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44 313 triglycerides, glucose and electrolytes.
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49 315 The whole procedure followed the clinical trial guidelines as required by the USA Food and
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51 316 Drug Administration, European Medicines Agency, and the Committee for Medicinal
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53 317 Products for Human Use.⁴⁴
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3 319 **Statistical analysis**
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5 320 Power calculations could not be done before the study because of the lack of information
6
7 321 from previous studies on potential effect sizes. In 2005 the prevalence of MS in Cyprus
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9 322 (600,000 population) was 120/100.000. Based on the aforementioned MS patients' numbers
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11 323 of our country and the centre of reference, the CING, we were able to enrol the 20% of the
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13 324 total RRMS patients eligible for treatment in the trial. Sample size was strictly based on this
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15 325 subjects' availability parameter and the novelty of the assessed intervention.
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21 327 Baseline characteristics were compared across all intervention groups by ANOVA or
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23 328 Kruskal-Wallis rank test for continuous variables and by mean Fisher's exact test for
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25 329 categorical variables, as appropriate.
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30 331 For the primary outcome, the ARR was analysed in a pair-wise fashion for the active
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32 332 interventions compared to placebo using negative binomial regression models adjusted for
33
34 333 number of relapses within two years before baseline, EDSS score at baseline and DMT. The
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36 334 relapse rate was calculated as the total number of relapses divided by the total number of
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38 335 patient-years followed for each treatment group. ARR differences were also calculated
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40 336 among all comparable parameters and reported as percent difference.
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45 338 For the secondary end-point outcome, the time to disability progression, Kaplan–Meier
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47 339 curves were constructed. Progression to disability and time thereof was compared in a pair-
48
49 340 wise fashion for the active interventions versus placebo by the log-rank test in the main
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51 341 analysis and by Cox proportional-hazards models with adjustment for baseline EDSS score,
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53 342 age and DMT in the supportive analysis. Each test was performed with a significance level of
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3 343 0.05. Multivariate models considered all variables with $P < 0.1$ on univariate models. There
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5 344 was no overt violation of the proportionality assumption.
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10 346 Both, per-protocol and intention to treat (ITT) analyses were performed, for different sets of
11
12 347 research questions to be answered, and both are reported. Missing data of the five lost to
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14 348 follow patients were imputed by use of the last-observation-carried-forward (LOCF)
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16 349 approach. Due to the proof-of-concept design of the study, the considerable non-adherence
17
18 350 rate (49%) and the interpretation issues caused thereof regarding the ITT analysis, the per-
19
20 351 protocol analysis considered being more informative and appropriate method approach to
21
22 352 answer the research addressed questions of efficacy of the interventions when subjects were
23
24 353 continuously following the protocol. All statistical analyses were well defined a priori. All
25
26 354 analyses were performed with STATA SE 10.0 (College Station, TX, USA). P-values are
27
28 355 two-tailed.
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33 34 357 **Role of the funding source**

35
36 358 The funders had no role in study design, data collection and analysis, decision to publish, or
37
38 359 preparation of the manuscript. All members of the writing group had full access to all study
39
40 360 data and contributed to its interpretation and prepared, reviewed, and approved the
41
42 361 manuscript for submission. All authors had final responsibility for the decision to submit the
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44 362 paper for publication.
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49 50 364 **Results**

51 52 365 **Study population**

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3 366 From July 2007 through December 2010 (including the 12-month extended period), a total of
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5 367 80 MS patients were randomly assigned to a study group at the CING (tertiary neurological
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7 368 center).
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11 370 Among the 80 patients, 20 patients were randomly assigned to each of the three groups to
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13 371 receive the interventions and 20 to receive placebo (Fig 1). Baseline characteristics of both
14
15 372 the ITT and the per-protocol populations were similar across groups (Table 2A and 2B). All
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17 373 patients that drop-out completed follow-up until study completion and were included in the
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19 374 ITT analyses (Table 4). Five patients were lost to follow before their first scheduled visit and
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21 375 two other patients that dropped-out before their first scheduled visit progressed to secondary
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23 376 progressive MS. Fifteen patients dropped-out without successfully completing the
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25 377 “normalization” period including five pregnancies. Another 17 patients dropped-out early
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27 378 after entry baseline. Seven patients that dropped out were given monoclonal antibody
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29 379 treatment (natalizumab). Overall, a total of 41 (51%) patients completed the 42-month study
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31 380 (July 2007 through December 31st 2010, including the 12-month extended period) where one
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33 381 patient from group A and two from the placebo group transferred on natalizumab, and 39
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35 382 (49%) patients either withdrew (drop-out) or lost to follow. Reasons for study-interventions
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37 383 discontinuation are listed in Figure 2.
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44 385 **Efficacy**

45 386 **Relapses**

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47 387 As a proof-of-concept trial we primarily needed to answer whether the interventions were
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49 388 effective for those MS patients who adhere to the assigned treatment, the per-protocol
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51 389 analysis.⁴⁵ For the sake of methodological comprehensiveness we also present the ITT
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53 390 analysis as a secondary analysis, to answer a different question, complementary to our core
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3 391 hypothesis; like what happened to MS patients who were placed on the interventions (the
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5 392 effect of assignment).⁴⁵
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10 394 Regarding the per-protocol analysis, during the first year of treatment, the ARR was 0.80,
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12 395 0.40, 0.78 and 0.83 for the four intervention groups respectively. During the second year, the
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14 396 ARR was 0.90, 0.40, 0.67 and 1.25 for the four intervention groups respectively. Overall, for
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16 397 the two-year primary end point, 8 relapses were recorded for the 10 patients in PLP10 group
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18 398 (0.40 ARR) versus 25 relapses for the 12 patients in placebo (1.04 ARR), a 64% adjusted
19
20
21 399 relative rate reduction (RRR) for the PLP10 group (RRR 0.36, 95% confidence interval (CI)
22
23 400 0.15 to 0.87, p=0.024) (Tables 3A, 5 and Fig 3A and 3C). Excluding patients on monoclonal
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25 401 antibody (natalizumab) treatment, the observed adjusted RRR became stronger (72%) over
26
27 402 the two years (RRR 0.28, 95% CI 0.10 to 0.79, p=0.016, Tables 3B and 5). Pair-wise
28
29 403 comparisons for the other two groups against placebo did not yield statistically significant
30
31 404 results (Tables 3A, 3B). The proportion of patients with ≤ 1 relapse for the two years on-study
32
33 405 was higher in the PLP10 group than in the placebo group (90% versus 42%, p=0.030, Table
34
35 406 5). Seeking to investigate further the observed difference, we compared the relapse rate
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37 407 during the 24 months before entry to the study to the 24 months on-treatment for each
38
39 408 intervention group. We observed a statistically significant relative reduction in the ARR
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41 409 (70%) only in the PLP10 group (RRR 0.30; 95% CI 0.14 to 0.65, p=0.003, Table 3A);
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43 410 within-group comparisons for the three other groups ARR reduction was not significant and
44
45 411 remained not significant when natalizumab treated patients were further excluded from the
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47 412 analysis. The effect of PLP10 through time at different time-windows versus placebo for all-
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49 413 time on-study patients is shown in Figures 3A to 3D. The ARR analysis, within time-
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51 414 windows, was not an assigned endpoint, but it could help in the process of evaluating parallel
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53 415 information as the time needed for a specific treatment intervention activity to be evident, as
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3 416 well as the efficacy profile through time. PLP10 reached maximum effect within a year on-
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5 417 treatment (counting from the entry baseline) and remained stable at an ARR of 0.4,
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7 418 displaying a steadily reduced ARR with long free-relapse time-windows. These group B
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9 419 characteristics are considered important parameters of a successful MS treatment where the
10
11 420 rule than the exception is the heterogeneity among patients' disease evolution. Specifically,
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13 421 Figure 3D demonstrates the dispersion of relapses throughout the 2-year period of all-time
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15 422 on-study (excluding patients on natalizumab) of PLP10 (n=10) versus placebo (n=10).
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17 423 Placebo, in line with the existing knowledge of how relapse history works in relation to future
18
19 424 relapses on MS patients (contagion phenomenon) indicates the expected linearly increased
20
21 425 trend of the relapse incidence.⁴⁶ The same phenomenon was true for the groups A and C.
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23 426 Finally, during the 12 month post-study extended period (January 1st 2010 to December 31st
24
25 427 2010) all-time on-study patients that received PLP10, showed persistent benefit in the ARR
26
27 428 compared to placebo (six relapses for the 10 subjects within PLP10, 0.6 ARR versus 19 for
28
29 429 the 12 subjects within placebo group, 1.58 ARR) indicating a statistically significant 62%
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31 430 adjusted relative rate reduction in the ARR for the PLP10 group (RRR 0.38, 95% CI 0.12 to
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33 431 0.99, p=0.046).
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433 Regarding the ITT analysis, within PLP10 group, none of the nine drop-out patients changed
434 to natalizumab and two out of nine discontinued because of pregnancy, whereas two out of
435 seven drop-out patients from the placebo group changed to natalizumab (a total of four
436 patients within the placebo arm population were on natalizumab, including the two patients
437 that transferred while all-time on-study versus none within PLP10 group (Supplementary
438 Information Fig 1). As reported, natalizumab reduces ARR by 68%, decreases the possibility
439 of disability progression by 43% with 57% of patients free of new or enlarging T2 lesions on
440 MRI scans compared to 15% on placebo.⁴⁷ The relapses of the drop-out patients are reported

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3 441 in Table 4A. As expected no statistically significant differences in the ARR were calculated
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5 442 for the comparison of any group versus placebo for the 24 months on-treatment, with a 0.75
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7 443 ARR within PLP10 (30 relapses) and 1.03 ARR within placebo (41 relapses) group, a 27%
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9 444 ARR reduction (Table 4B). Interestingly, despite the high non-adherence rate, there was a
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11 445 statistically significant difference for the comparison of the ARR in the 24 months before
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13 446 entry baseline to the 24 months on-treatment for the PLP10 group (RRR 0.45, 95% CI 0.26 to
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15 447 0.78, $p=0.005$).

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449 **Disability progression**

450 Regarding the per-protocol analysis, at two years, the time to disability progression, with
451 confirmation after six months (secondary end-point) was significantly longer only with
452 PLP10. The cumulative probability of disability progression was 10% in the PLP10 group
453 and 58% in the placebo group ($p=0.019$) (Supplementary Information Fig 2). After excluding
454 patients on natalizumab, there was an increased statistically significant difference between
455 the PLP10 and the placebo group for the same analysis ($p=0.006$) (Fig 4A). At two years, the
456 cumulative probability of progression was 10% in the PLP10 group and 70% in the placebo
457 group, which represents a decrease of 60 percentage points or a relative 86% decrease in the
458 risk of sustained progression of disability within PLP10 group (adjusted hazard ratio, 0.11;
459 95% CI 0.01 to 0.97, $p=0.047$). One versus seven out of ten patients progressed to confirmed
460 disability in the PLP10 and the placebo groups respectively when patients on natalizumab
461 were excluded. No statistically significant difference was observed for any comparison of the
462 other two groups compared to placebo (Fig 4A and Supplementary Information Fig 2).

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464 Regarding the ITT analysis, at two years, the cumulative probability of progression was 10%
465 in the PLP10 group and 35% in the placebo group ($p=0.052$, a trend for an effect), which

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3 466 represents a decrease of 25 percentage points or a relative 71% decrease of the PLP10 for the
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5 467 risk of sustained progression of disability (adjusted hazard ratio 0.22, 95% CI 0.04 to 1.07,
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7 468 $p=0.06$) (Fig 4B). Two versus seven out of the total randomized patients progressed to
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9 469 confirmed disability in the PLP10 and the placebo groups respectively. No significant
10
11 470 differences were observed for groups A or C against placebo (Fig 4B). The mean change in
12
13 471 Expanded Disability Status Scale (EDSS) score as a function of visit number is shown in
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15 472 Figure 5.
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21 474 **MRI**

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23 475 Over two years, the MRI results support the overall conclusion from the study that PLP10 has
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25 476 a positive effect on disease activity since only 29% from the PLP10 group as opposed to 67%
26
27 477 from the placebo group developed new or enlarging T2 lesions (57% relative risk reduction).
28
29 478 Excluding patients on natalizumab there is an increased relative risk reduction (64%) between
30
31 479 PLP10 as opposed to placebo with 29% of patients on PLP10 and 80% on placebo with
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33 480 development of new or enlarging T2 lesions (Table 5).
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38 482 **Safety**

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40 483 Over the course of the 30 month study no significant adverse events were reported from any
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42 484 group. According to a questioner procedure the only aetiology for drop-outs was the
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44 485 palatability and smell of the formula preparations. Nausea was reported by two patients. No
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46 486 abnormal values observed on any of the biochemical and haematological blood tests. No
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48 487 allergic reactions reported.
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54 489 **Discussion**

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3 490 In this proof-of-concept clinical trial assessing the safety and efficacy of three cocktail
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5 491 intervention formulas in RRMS, we observed a significant benefit for the novel PLP10
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7 492 intervention compared to placebo for both the ARR and the progression to disability. Our
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9 493 results include analyses pertaining to a total of 42 months study collected data, including the
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11 494 12-month, free of intervention treatment, extension period. We focused on the per-protocol
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13 495 data analysis since it is the appropriate method to best provide the answer to the proof-of-
14
15 496 concept trial-addressed question. The high drop-out rate was mostly the result of formulas
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17 497 palatability, a common phenomenon in trials using oily interventions where a lot of patients
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19 498 tend to drop-out soon after first dosage. We thus present our main per-protocol analysis, as
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21 499 well as a subgroup analysis excluding patients on natalizumab. We have found a statistically
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23 500 significant reduction in the ARR and the disability progression comparing not only patients
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25 501 on PLP10 versus placebo but also comparing the ARR of the PLP10 patients in the 24-month
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27 502 period prior to the study to the ARR of the 24 months on-study; the observed differences
28
29 503 became larger when patients that received natalizumab (the most potent disease modifier)
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31 504 were excluded. The ARR decreased within a year on PLP10 and significantly remained stable
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33 505 until study completion. Statistically significant difference of ARR between patients on PLP10
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35 506 versus placebo continued for the additional 12 month extended period (persistent effect)
36
37 507 without significant difference on DMT. These clinical findings are supported by the results
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39 508 regarding the MRI analysis where the proportion of patients free from new or enlarging brain
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41 509 T2 lesions was also higher in PLP10 group versus placebo. The persistent effect within the
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43 510 extended period it is considered of major importance and supportive of the results since it is
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45 511 in agreement with the very long washouts, reported necessary, for omega-3 fatty acids and
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47 512 especially DHA to return towards pretreatment values within the fatty acids of plasma,
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49 513 platelets, monocytes and red blood cells.⁴² This study also provides important 30-month,

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3 514 placebo-controlled information about the safety of PLP10, A and C interventions. No severe
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5 515 side effects have been reported.
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9 517 As medications used to treat MS become increasingly highly specific and potent, attention to
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11 518 safety is paramount. Current available treatments are products of reductionism, partially
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13 519 effective, associated with severe side effects without (re)myelinating or neuroprotective
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15 520 abilities. Interferons and glatiramer acetate, the most widely used first-line MS drugs
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17 521 available today, are associated with the least severe side-effects among MS therapies but they
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19 522 are reported with only 29-33% ARR reduction and with no significant effects on the
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21 523 progression of disability. Natalizumab as previously discussed and Fingolimod with 54%
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23 524 ARR reduction (without significant benefit on the progression of disability) are second-line
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25 525 drugs associated with severe side-effects.^{47, 48}
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32 527 No existing MS treatment has ever been designed as a result of SM concept approach or with
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34 528 a potential to effectively stimulate intrinsic remyelinating and neuroprotecting mechanisms or
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36 529 exert such an action. Now we propose that a holistic SM model approach has to be applied by
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38 530 synchronized action on all involved perturbed mechanisms. PLP10 has innovative
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40 531 characteristics with a postulated efficacy attained through different mechanisms of action and
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42 532 probably by the synergistic effect of its constituent ingredients. PLP10 has all the
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44 533 characteristics of a medical food with the action to feed a normal metabolic process by
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46 534 supplying nutritional structural membrane precursors, building blocks, and vitamins from
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48 535 dietary sources that enhance remyelination and neuroprotection and simultaneously promote
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50 536 normalization of all cellular membranes lipid content. The intention is to normalize the
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52 537 specific nutritional requirements of the MS patients.
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3 539 Different factors and molecular entities appear to be part of the possible aetiology for MS
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5 540 with specific PUFA and antioxidants found to be key substances related to all known
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7 541 pathogenic and recovery mechanisms. But, it is well established that MS patients are
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9 542 characterized with significant deficiencies in antioxidant efficiency as well as in PUFA levels
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11 543 in blood and cellular membranes.^{11, 49-51}
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16 545 According to one hypothesis, the change in the ratio of omega-3 to omega-6, due to
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18 546 increasing consumption of omega-6 PUFA, meaning high accumulation of AA, in the
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20 547 Western diet, may be one of the major factors responsible for the increasing incidence of
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22 548 inflammatory diseases relative to populations. In the Western diet, the ratio of omega-3 to
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24 549 omega-6 is about 1:20–30; in populations that consume fish-based diets, the ratio is about
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26 550 1:1–2.^{52, 53} The intervention daily dose was aiming and believed to be high enough to
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28 551 restore/amplify body efficient antioxidant activity and ensure cellular membranes lipid profile
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30 552 normalization (PUFA content) and simultaneously potentiate involvement of the ingredients
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32 553 in the anti-inflammatory and recovery mechanisms. Diet fatty acid molecules need about six
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34 554 months period to exert their beneficial effect and this essential parameter was for the first
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36 555 time under consideration in our study design (normalization period).⁴² This chronotherapy
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38 556 parameter it is of major importance in line with the SM treatment philosophy and if it is not
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40 557 included in the trial design the possibility of misleading result evaluation greatly increases. In
41
42 558 fact, considering that omega-3 supplementation can release and replace excess AA within the
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44 559 cellular membranes, we can speculate that an increased inflammatory activity can possibly
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46 560 result during the first six months of supplementation (during normalization period).
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54 562 The maintenance of myelin requires continued turnover of its components throughout life.^{54,55}
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56 563 In addition to the EPA, DHA, LA, and GLA, PLP10 was containing limited quantities of
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3 564 other structural PUFA, specific MUFA (mostly oleic acid) and SFA (palmitic and stearic
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5 565 acid), specifically to provide a direct source for neuronal cell membranes rehabilitation and
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7 566 for (re)myelination and neuroprotection since they are all major components, precursors and
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10 567 building blocks of any new physiological myelin and cellular membranes in general.
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12 568 Assembly of the correct molecules into myelin membrane may be especially critical during
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14 569 active synthesis. Possibly, if critical constituents aren't available or are metabolically
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16 570 blocked, amyelination, dysmyelination or demyelination may ensue.⁵⁶
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21 572 The well known and established safety of the ingredients used and the protocol guidelines
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23 573 were supportive reasons for us to proceed with the clinical study even though with limitation
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25 574 on the pre-estimation of required trial sample size as it was discussed in method section. The
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27 575 adherence of the subjects is another issue but the duration of the study (42 months) is adding
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29 576 power to the results;⁴⁴ having the research questions been consciously and carefully
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31 577 approached and answered. Furthermore, the statistical methodologies used along with the
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33 578 appropriate adjustments, broadly accepted for MS clinical trials, power the findings, results,
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35 579 and significance. The baseline characteristics of the treatment arms could possibly be
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37 580 considered indicative of four very active groups of patients but that was the result of the
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39 581 limited number of RRMS population eligible for the study within Cyprus. On the other hand
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41 582 the balanced baseline characteristics without statistical differences, the statistical adjustments
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43 583 (for all important baseline parameters i.e. relapses, EDSS, age and DMT) and the
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45 584 randomization within four different groups are the safety valves against data
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47 585 misinterpretation. Yet, in small randomized control trials with a high drop-out rate, the per-
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49 586 protocol analysis could be affected by the characteristics of the patients dropping out. In
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51 587 order to safeguard our findings in the best possible way under the circumstances, we
52
53 588 proceeded to adjusting for confounders. Moreover, we cannot discard our finding as a false
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3 589 positive, given that this is a randomized, double-blind, placebo-controlled clinical trial and,
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5 590 despite its small sample size, represents a piece of evidence that only a larger randomized
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7 591 controlled trial can replicate or refute. It is possible to question why DMTs efficacy cannot
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9 592 be emerged out of the data analysis, of the four treatment arms, and in accordance to their
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11 593 published values. We believe that the limited efficacy of the DMTs, the sample size and the
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13 594 statistical adjustments were strong limiting determining factors for such an indication to be
14
15 595 countable. An additional argument is that the efficacy reported for the analysis of pre-
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17 596 treatment (24 months before entry baseline) versus on-trial ARR could be considered as
18
19 597 potentially biased due to differences of how relapses were defined during the course of a
20
21 598 study compared to pre-treatment period; or due to regression to the mean or placebo effect.
22
23 599 This analysis was performed as an additional exploratory analysis that we were able to do due
24
25 600 to the availability of data. The relapses of the two pre-treatment years were drawn out of the
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27 601 patients' archival records by the same treating neurologist involved in the study (MP), and
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29 602 according to the patients' hospitalization date for receiving intravenous methyl-prednisolone.
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31 603 This analysis was not used as a primary or a secondary end-point under investigation
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33 604 although it is usually reported by many clinical studies. As a matter of fact many early phase
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35 605 trials are based only on such an analysis (before versus after treatment results). In almost all
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37 606 MS trials the number of relapses within the two years before baseline is a factor under
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39 607 adjustment for the statistical analyses.⁴⁸ The inclusion of the post-hoc MRI analysis is another
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41 608 limiting factor that needs attention since it was used as an additional aside exploratory
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43 609 approach (due to study budget limitations it was not possible to be used as a formal
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45 610 endpoint); but the MRI evaluation was blinded and can be considered as representative of the
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47 611 randomized subjects within the treatment arms. As far as the regression to the mean and the
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49 612 placebo effect concerns we believe that the 6-month normalization period is an accountable
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51 613 and valuable eliminating factor of the possible effect; as well as the presence of four groups,
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3 614 where only the PLP10 treatment arm is associated with statistically significant efficacy versus
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5 615 placebo.

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10 617 Our observations are consistent with the idea that simultaneous availability of specific PUFA
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12 618 along with other major membrane and myelin building blocks in combination with specific
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14 619 antioxidants, within optimum quantity, quality, ratio and structural form can possibly result to
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16 620 a more appropriate holistic therapy reducing MS disease activity. This is probably succeeded
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18 621 through synergistic and/or simultaneous effect on the interactions and dynamics of the most
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20 622 probable environmental and biological disease causing factors that induce complex biological
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22 623 network of events for disease pathogenesis and evolution; as well as on the protective and
23
24 624 reparative mechanisms. We can additionally speculate that the nature of the intervention
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26 625 formula cannot be prohibitive for its use as preventive regimen and does not preclude
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28 626 probable positive efficacy on the other types of MS, but has to be further investigated. A
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30 627 larger size multicenter clinical trial will better establish PLP10 place in the armamentarium of
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32 628 treatments for MS.

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38 630 It is commonly accepted that nutrition is one of the possible environmental factors involved
39
40 631 in the pathogenesis of MS, but its role as complementary MS treatment is unclear and largely
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42 632 disregarded.⁵⁷ It is well known that the majority of the patients suffering from MS they do
43
44 633 use dietary supplements for a variable length of time and they prefer supplement type of
45
46 634 “help” over conventional drugs.⁵⁸ Dietary antioxidants and fatty acids may influence the
47
48 635 disease process in MS by reducing immune-mediated inflammation, oxidative stress and
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50 636 excitotoxic damage.¹¹ Present data reveal that healthy dietary molecules have a pleiotropic
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52 637 role and are able to change cell metabolism from anabolism to catabolism and down-regulate
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54 638 inflammation by interacting with enzymes, nuclear receptors and transcriptional factors.⁵⁷

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3 639 The present preliminary small size randomized controlled phase II clinical trial, for the first
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5 640 time provides link evidence between dietary, metabolic, immunological, and neurobiological
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7 641 aspects of MS after three quarters of a century of unsuccessful scientific efforts. This link
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9 642 evidence might probably be the beginning of opening new horizons and new avenues in the
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11 643 approach of MS prevention and treatment, and possibly of other multifactorial chronic
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13 644 diseases, including neurodegenerative and autoimmune as well.
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1.
Treatment Arms

A†	B (PLP10)†	C†	Placebo†
Intervention: EPA (1,650mg) / DHA (4,650mg) / GLA (2,000mg) / LA (3,850mg) / total other omega-3 (600mg)* / total MUFA** (1,714mg) + total SFA (18:0 160mg, 16:0 650mg) / vitamin A (0.6mg) / vitamin E (22mg) plus citrus aroma	Intervention: EPA (1,650mg) / DHA (4,650mg) / GLA (2,000mg) / LA (3,850mg) / total other omega-3 (600mg)* / total MUFA** (1,714mg) + total SFA (18:0 160mg, 16:0 650mg) / vitamin A (0.6mg) / vitamin E (22mg) + pure γ -tocopherol (760mg) plus citrus aroma	Intervention: pure γ -tocopherol (760mg) dispersed in pure virgin olive oil (16,137 mg) as delivery vehicle plus citrus aroma	Intervention: Olive oil (pure virgin) plus citrus aroma

* Other omega-3: C18:3n-3 37mg, C18:4n-3 73mg, C20:4n-3 98mg, C22:5n-3 392mg
 ** MUFA: 18:1 1300mg, 20:1 250mg, 22:1 82mg, 24:1 82mg
 † Total daily dose 19.5ml

EPAX1050, EPAX AS, Aalesund, Norway; was used as the source for the omega-3 PUFA, as re-esterified glycerides from fish body oils; Borage seed oil (organic, cold pressed) “Borago officinalis” Goerlich Pharma International GmbH, Edling, Germany, was used as the source for the omega-6 PUFA, MUFA and SFA, as triglycerides. The pure γ -tocopherol was purchased from Tama Biochemical Co. Ltd., Shinjuku-ku Tokyo, Japan; vitamin A as beta-carotene from HealthAid Ltd., Middlesex, United Kingdom and the Citrus aroma from Givaudan Schwaiz AG, Dubendorf, Switzerland.

Table 1. Intervention ingredients per treatment arm.

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2A.					
Characteristics	Group A (n=20)	Group B† (n=20)	Group C (n=20)	Placebo (n=20)	P- value
Sex					
Female - no. (%)	15 (75)	15 (75)	15 (75)	15 (75)	1.000
Age (yr)					
Mean ± Standard Deviation (SD)	38.0±11.9	36.9±8.4	37.7±8.7	38.1±10.9	0.982
Median (Range)	38.0 (22–65)	37.0 (25–61)	36.5 (24–54)	36.0 (21–58)	
Treatment history					
Patients on DMT- no. (%)	11 (55)	9 (45)	12 (60)	10 (50)	0.875
Pre-treatment disease duration (yr)					
Mean ± SD	9.0±7.6	8.6±4.8	8.6±5.3	7.7±5.7	0.909
Median (Range)	7.5 (2–37)	8.0 (2–20)	8.0 (3–24)	6.5 (2–25)	
Pre-treatment relapses‡					
Mean ± SD	2.33±1.68	2.41±1.73	2.31±1.66	2.10±1.32	0.946
Median (Range)	2.0 (1–6)	2.0 (1–7)	2.0 (1–6)	2.0 (1–4)	
ARR	1.17	1.21	1.16	1.05	0.946
Patients -% with ≤1 relapse	40	45	40	35	
Baseline EDSS score‡					
Mean ± SD	2.52±1.23	2.15±1.05	2.42±1.21	2.39± 0.93	0.775
Median (Range)	2.5 (1.0–5.5)	2.0 (1.0–4.0)	2.5 (0.0–5.0)	2.5 (1.0–4.0)	
2B.					
Characteristics	Group A (n=10)	Group B† (n=10)	Group C (n=9)	Placebo (n=12)	P- value
Sex					
Female - no. (%)	5 (50)	7 (70)	6 (66.6)	10 (83.3)	0.419
Age (yr)					
Mean ± SD	36.6±13.5	34.80±5.4	40.9±8.1	39.8±13.2	0.572
Median (Range)	34.5 (22–65)	34.5 (26–43)	40.0 (29–54)	37.5 (21–58)	
Treatment history					
Patients on DMT- no. (%)	6 (60)	4 (40)	6 (67)	6 (50)	0.949
Pre-treatment disease duration (yr)					
Mean ± SD	9.7±10.0	8.3±5.3	11.3±6.1	8.7± 7.1	0.807
Median (Range)	7.5 (2–37)	8.0 (2–20)	8.0 (4–24)	5.5 (2–25)	
Pre-treatment relapses					

Mean ± SD	2.20±1.47	2.70±1.25	1.78±0.66	1.67±1.37	0.241
Median (Range)	2.0 (1 – 6)	2.5 (1 – 4)	2.0 (1 – 3)	1.5 (1 – 4)	
ARR	1.10	1.35	0.89	0.83	
Patients -% with ≤1 relapse	30	20	33	50	
Baseline EDSS score					
Mean ± SD	2.65±1.37	2.40±1.12	2.11±1.02	2.16±0.96	0.698
Median (Range)	3.0 (1.0–5.5)	2.5 (1.0–4.0)	2.0 (1.0–4.0)	2.0 (1.0–3.5)	

† PLP10 group

‡ Available data at Entry Baseline (n=18 for group A, n=17 for group B, n=19 for group C, n=19 for group D)

Table 2. The table section 2A reports the demographics and baseline disease characteristics for total randomized population by treatment arm.

The table section 2B reports the demographics and baseline disease characteristics of all-time on-study population by treatment arm. There were no significant between study-group differences at baseline for any characteristic.

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3A.								
Characteristics	Group A (N=10)		Group B† (N=10)		Group C (N=9)		Placebo (N=12)	
End Point	X	Y	X	Y	X	Y	X	Y
Total No. of relapses	22	17	27	8	16	13	20	25
Annual Relapse Rate (ARR)	1.10	0.85	1.35	0.40	0.88	0.72	0.83	1.04
% Reduction Compared to Placebo (primary endpoint) (Ys)¶	-18		-62		-30		N/A	
P Value against placebo	0.468		0.024		0.578			
ARR change -% (Y to X)¶	-23		-70		-18		+25	
P value against baseline	0.425		0.003		0.578		0.500	
X: Total number of relapses of 24 months pre-treatment (baseline) Y: Total number of relapses of 24 months on-treatment ¶ Unadjusted estimate								
3B.								
Excluding patients on natalizumab	Group A (N=9)		Group B† (N=10)		Group C (N=9)		Placebo (N=10)	
End Point	X	Y	X	Y	X	Y	X	Y
Total No. of relapses	16	15	27	8	16	13	13	19
Annual Relapse Rate (ARR)	0.88	0.83	1.35	0.40	0.88	0.72	0.65	0.95
% Reduction Compared to Placebo (primary endpoint) (Ys)¶	-13		-58		-24		N/A	
P Value against placebo	0.493		0.016		0.412			
ARR change -% (Y to X)¶	-6		-70		-18		+46	
P value against baseline	0.857		0.003		0.578		0.354	
X: Total number of relapses of 24 months pre-treatment (baseline) Y: Total number of relapses of 24 months on-treatment † PLP10 group ¶ Unadjusted estimate								
Table 3. The table section 3A reports the two year primary end points of ARR of all-time on-study population by treatment arm and percent difference with placebo. During the 24mo period on-treatment								

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3 the ARR of the group A was 0.85 with 18% decrease compared to placebo (p=0.468), of PLP10 group
4 0.40 with 62% decrease (p=0.024), and of group C 0.72 with 30% decrease (p=0.578) and reports the
5 comparison of the 24mo pre-treatment ARR (baseline ARR) to 24mo on-treatment ARR of all-time on-
6 study population including patients on natalizumab.
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14 The table section 3B reports comparison of the 24mo pre-treatment ARR to 24mo on-treatment ARR of
15 all-time on-study population excluding patients on natalizumab; and the comparison of the ARR during
16 the 24mo period on-treatment (primary end point) between each one of the groups against placebo.
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4A.									
Characteristics	Group A (N=8)		Group B† (N=7)		Group C (N=10)		Placebo (N=7)		
	X	Y	X	Y	X	Y	X	Y	
No. of Relapses	20	14	14	14	27	26	20	13	
Annual Relapse Rate (ARR)	1.25	0.88	1.00	1.00	1.35	1.30	1.42	0.92	
X: Total number of relapses of 24 months pre-treatment Y: Total number of relapses of 24 months on-treatment									
4B.									
Characteristics	Group A (N=20)		Group B† (N=20)		Group C (N=20)		Placebo (N=20)		
End Point	X	Y	X	Y	X	Y	X	Y	
No. of Relapses	45	34	49	30	46	41	43	41	
Annual Relapse Rate (ARR)	1.13	0.85	1.23	0.75	1.15	1.03	1.08	1.03	
ARR Reduction -% (Y to X)¶	-25		-39		-10		-5		
P value against baseline	0.120		0.005		0.475		0.652		
% Reduction of the ARR Compared to Placebo (Ys)¶	-18		-27		0.0		N/A		
P Value against placebo	0.447		0.121		0.996				
X: Total number of relapses of 24 months pre-treatment (baseline) Y: Total number of relapses of 24 months on-treatment ¶ Unadjusted estimate † PLP10 group									

Table 4. The table section 4A reports the two year primary end point of relapses based on study design as reported by drop-out patients by Treatment Arm. The most drop-out patients that went on disease modified therapy (DMT) were from the group A and placebo with three and two patients respectively on natalizumab. These parameters justify the decreased number of relapses recorded within group A and placebo drop-outs; and this could affect the ITT analysis in favor of placebo when the total two-year recorded data are used. For PLP10 group, 14 relapses were reported at baseline and remained the same during the two-year study. For placebo, 20 relapses were reported at baseline and decreased to 13 during the two-

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3 year study. This is expected since, for PLP10 group 43% of the drop-outs were under DMT at
4 entry baseline and remained the same until the end of the study with no patient on
5 natalizumab; but 57% of the placebo group drop-outs that were under DMT at entry baseline
6 increased to 86% at the end of the study including two patients on natalizumab.
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14 The table section 4B reports comparison of 24 months pre-treatment ARR (baseline) to 24
15 months on-treatment ARR of total randomized population, by treatment arm. The ARR of
16 PLP10 group was 1.23 at baseline and 0.75 at the end of the study (39% reduction $p=0.005$),
17 and for placebo 1.08 at baseline and 1.03 at the end of the study (5% reduction $p=0.652$). No
18 statistical difference was calculated for the other two treatment arms. During the 24 months
19 on-treatment, PLP10 group presented a 27% reduction of ARR versus placebo ($p=0.121$),
20 with all groups without statistically significant results.
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5.					
Characteristics*	Group A (n=10)	Group B PLP10 (n=10)	Group C (n=9)	Placebo (n=12)	P-value Group B vs. Placebo
Annual relapse rate over 1 year**	0.80	0.40	0.78	0.83	
Total number of relapses**	8	4	7	10	
Primary end points					
Annual relapse rate over 2 years (95% CI)**	0.85	0.40 (0.15-0.87)	0.72	1.04	0.024
Total number of relapses**	17	8	13	25	
Excluding patients on natalizumab					
Annual relapse rate over 2 years (95% CI)	0.83	0.40 (0.10-0.79)	0.72	0.95	0.016
Total number of relapses	15	8	13	19	
Secondary end points					
Cumulative probability of sustained progression increase by 1 point on EDSS confirmed after 6 mo, over 2 years -% **	43	10 (1/10)	24	58 (7/12)	0.019
Excluding patients on natalizumab					
cumulative probability of sustained progression increase by 1 point on EDSS confirmed after 6 mo, over 2 years -%	33	10 (1/10)	24	70 (7/10)	0.006
Exploratory Results					
Patients proportion with ≤1 relapse over 2 years -% **	50 (5/10)	90 (9/10)	56 (5/9)	42 (5/12)	0.030
MRI					
Patients proportion with new or enlarging T2 lesions-% **	----	29 (2/7)	----	67 (4/6)	
Excluding patients on natalizumab					
Patients proportion with no new or enlarging T2 lesions-%	----	29 (2/7)	----	80 (4/5)	
DMT (interferons, glatiramer acetate) and natalizumab					
Patients proportion on DMT and natalizumab at the end of 2 years-% **	80 (8/10)†	60 (6/10)	67 (6/9)	75 (9/12) ‡	0.747
* CI denotes confidence interval.					
** Including patients on natalizumab					
† 1 out of 10 on natalizumab					
‡ 2 out of 12 on natalizumab					

Table 5. Clinical end points, according to study group for all-time on-study population.

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22
23 727 exchange of investigating, on their behalf, the efficacy and safety of γ -tocopherol.
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30
31 730 authors critically revised and approved the final version. M.C.P and I.S.P were responsible
32
33 731 for the protocol and study design. M.C.P was the evaluator of patients' clinical progress signs
34
35 732 and the treatment physician. I.S.P and G.N.L were involved in the non-pharmacologic
36
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38
39 734 contributed on the intervention formulation and composition rational. I.S.P supervised the
40
41 735 composition procedure of the interventions and the fatty acid profile analysis of the red blood
42
43 736 cell membranes. E.E.N and I.S.P performed the statistical analyses. E.E.N was an
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47 738 the statistical analyses.
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7 742 the business incubator.
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27 751 No pharmaceutical companies were involved in this phase II clinical trial. The intervention is
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29 752 under a USA provisional patent; Application Number 61469081.
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34 754 Ethical approval: Cyprus National bioethics committee (reference EEBK/EII/2005/10).
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38 756 All authors have completed the Unified Competing Interest form at
39
40 757 www.icmje.org/coi_disclosure.pdf (available on request from the corresponding author) and
41
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49
50 762 Ltd. that might have an interest in the submitted work in the previous 3 years; (3) their
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52 763 spouses, partners, or children have no financial relationships that may be relevant to the
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3 764 submitted work; and (4) E.E.N has a non-financial interests that may be relevant to the
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Article Summary

Article focus:

- The increasing prevalence of Multiple Sclerosis (MS), the limited efficacy and the side effects of the existing treatments urge the clinical need for the development of new, innovative, more effective, safe, and preventive treatment strategies.
- For the first time we propose three original nutraceutical treatment intervention-cocktails, formulated based on systems medicine through nutritional systems biology rational; with PLP10 representing the complete composition of the formulation and the other two treatments represent partial formulations.
- We have studied the proposed interventions on 80 relapsing remitting MS patients (four groups of 20 each) for a total of 42 months, in a randomized, double-blind, placebo-controlled, phase II proof-of-concept clinical trial.

Key messages:

- The per-protocol data analyses indicated that PLP10 is associated with significant efficacy versus placebo on both reducing the annualized relapse rate and disease progression without any adverse or severe side effects.
- Overall, for this small size phase II study, a total of 41 (51%) patients completed the 42-month trial. For the per-protocol analysis of the two-year primary end point, 8 relapses were recorded in the PLP10 group (n=10) (0.40 ARR) versus 25 relapses in the placebo group (n=12) (1.04 ARR), a 64% adjusted relative rate reduction for the PLP10 group (RRR 0.36, 95% CI 0.15 to 0.87, p=0.024). In a subgroup analysis that excluded patients on monoclonal antibody (natalizumab) treatment, the observed adjusted RRR became stronger (72%) over the two years (RRR 0.28, 95% CI 0.10 to 0.79, p=0.016). Per-protocol analysis for the secondary outcome at two years, time to disability progression, was significantly longer only with PLP10. The cumulative probability of disability progression at two years was 10% in the PLP10 group and 58% in the placebo group (unadjusted log-rank p=0.019). In a subgroup analysis that excluded patients on natalizumab the cumulative probability of progression was 10% for the 10 patients in the PLP10 group and 70% for the 12 patients in the placebo group, a relative 86% decrease in the risk of sustained progression of disability in the PLP10 group (unadjusted log-rank p=0.006; adjusted hazard ratio, 0.11; 95% CI 0.01 to 0.97, p=0.047).
- PLP10 has probably the ability to interfere with the total known repertoire of mechanisms involved in MS pathogenesis as well as with the recovery mechanisms, neuroprotection and remyelination.; it is potentially able to manipulate global perturbed networks of the disease causing factors rather than focusing only on unique failing components.
- This proof-of-concept clinical study might be indicative and the beginning of new avenues in MS prevention and treatment and of new epoch of novel drug development

with dynamic therapeutic potential for chronic complex multifactorial diseases.

Strengths and limitations of this study:

- The strength of this study is the systems medicine therapeutic approach, the length of the study the inclusion of the six months normalization (chronotherapy) period, and the study protocol following all indicated appropriate guidelines with definite inclusion/exclusion criteria and primary/secondary end points.
- The sample size and the high rate of drop-outs (palatability of the formula) are the limitations associated with the present study.
- A phase III multi-center clinical trial will establish the present intervention regime among the best choices among neurodegenerative diseases prophylaxis and MS treatment drugs faretra.

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3 927 **Figure legends**
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6 928 **Figure 1.** Study Flowchart
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9 929 **Figure 2.** Omega-6 and omega-3 PUFA, their respective metabolic derivatives and their
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11 possible effects on inflammation.
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15 931 After consumption, the PUFAs are metabolized via several pathways (not shown) to active
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17 932 compounds that mediate inflammation and products that promote resolution of inflammation.
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20 933 Abbreviations: PL, phospholipid; IFN- γ , interferon γ ; IL-2, interleukin 2; NF κ B, nuclear
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22 934 factor kappa B; PGE2, prostaglandin E2; PPAR γ , peroxisome proliferator-activated receptor
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24 935 γ ; PUFAs, polyunsaturated fatty acids; TGF β , transforming growth factor β ; TNF, tumor
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26 936 necrosis factor; COX, cyclooxygenase; hydroxyeicosatetraenoic acid; HPETE,
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28 937 hydroperoxyeicosatetraenoic acid; LOX, lipoxygenase; LT, leukotriene; PG, prostaglandin;
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30 938 TX, thromboxane; RXR- γ , retinoid X receptor-gamma; Nrf2, nuclear respiratory factor;
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32 939 MMP, metalloproteinase.
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37 940 **Figure 3.** Panel A demonstrates the ARR of all-time on-study patients during the 24mo pre-
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39 941 treatment (baseline ARR) and at different on-study intervals (6, 12, 18, 24 mo) per treatment
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41 942 arm. **
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44 943 Panel B demonstrates the ARR of all-time on-study population between 0-6, 6-12, 6-18, and
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46 944 6-24 mo period intervals, of PLP10 vs. placebo group. **
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49 945 Panel C demonstrates the ARR of all-time on-study population of PLP10 vs. placebo group at
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51 946 baseline, during 1st year, and during the 2-year on-treatment. **
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55 947 Panel D demonstrates the dispersion of relapses throughout the 2-year period of all-time on-
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57 948 study (excluding patients on natalizumab) of PLP10 (n=10) vs. placebo (n=10). Placebo
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3 949 shows an irregular dispersion of relapses in relation to PLP10 group with linear increasing
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5 950 trend while PLP10 shows a stabilized linear trend. By using the per-protocol model where
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7 951 patients on natalizumab were excluded, we could compare the number of relapses on a same
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9 952 number of patients.

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13 953 ** Including the patients on natalizumab.

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16 954 **Figure 4.** Panel 4A demonstrates the Kaplan–Meier plot of the time to sustained progression
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18 955 of disability among all-time on-study patients, excluding patients on natalizumab, receiving
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20 956 intervention A, PLP10 and C as compared with placebo. PLP10 reduced the risk of sustained
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22 957 progression of disability by 86% over two years (p=0.006). Intervention formula A reduced
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24 958 the risk of sustained progression of disability by 53% (p=0.266) and intervention formula C
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26 959 by 67% (p=0.061).

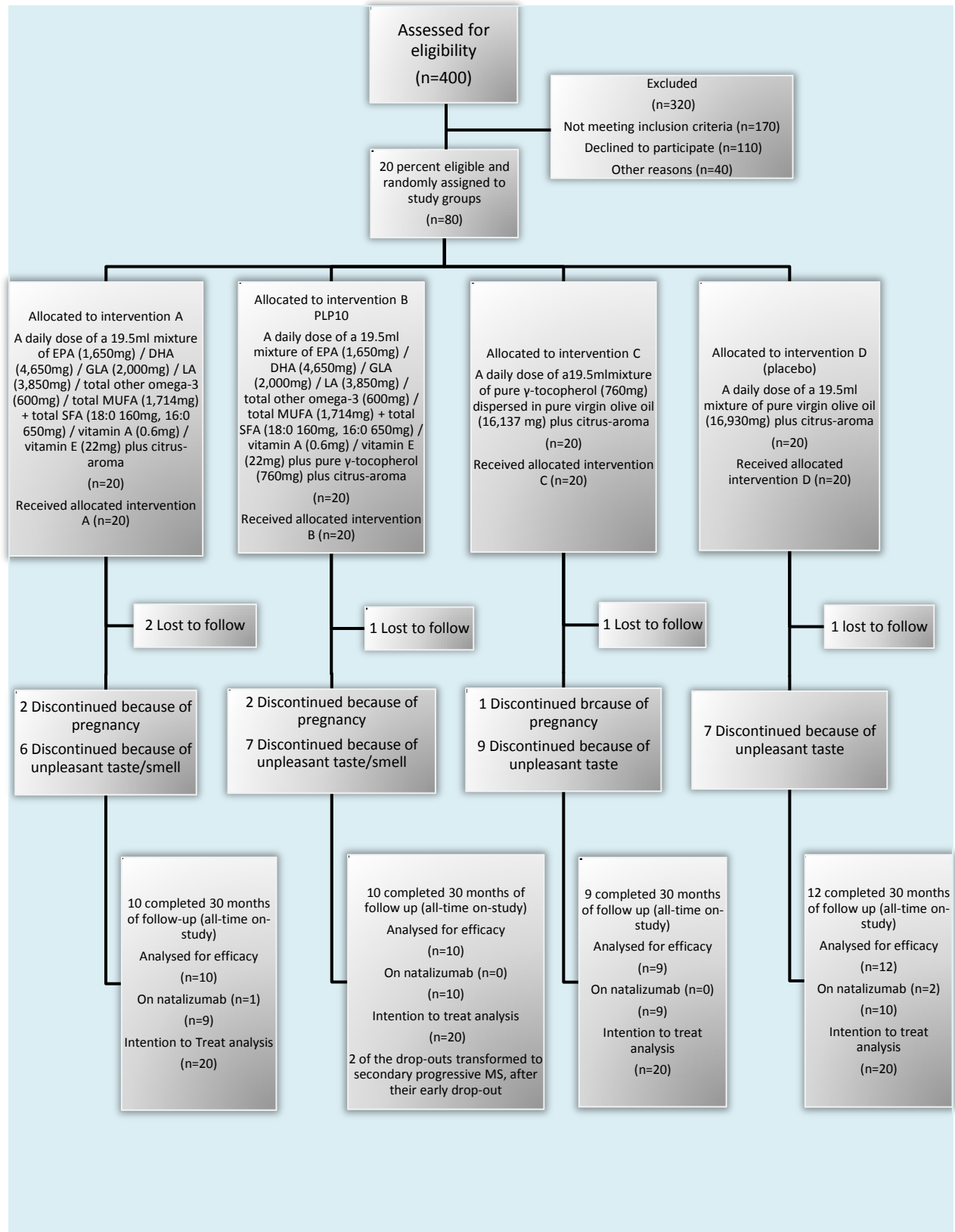
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30 960 Panel 4B demonstrates the Kaplan–Meier plot of the time to sustained progression of
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32 961 disability among ITT population receiving intervention A, PLP10 and C as compared with
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34 962 placebo. PLP10 reduced the risk of sustained progression of disability by 71% over two years
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36 963 (p=0.052, trend). Intervention formula A reduced the risk of sustained progression of
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38 964 disability by 22% (p=0.727) and intervention formula C by 40% (p=0.447).

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41 965 **Figure 5.** Mean change in expanded disability status scale score as a function of visit
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43 966 number. Values are expressed as mean ± s.e.m.

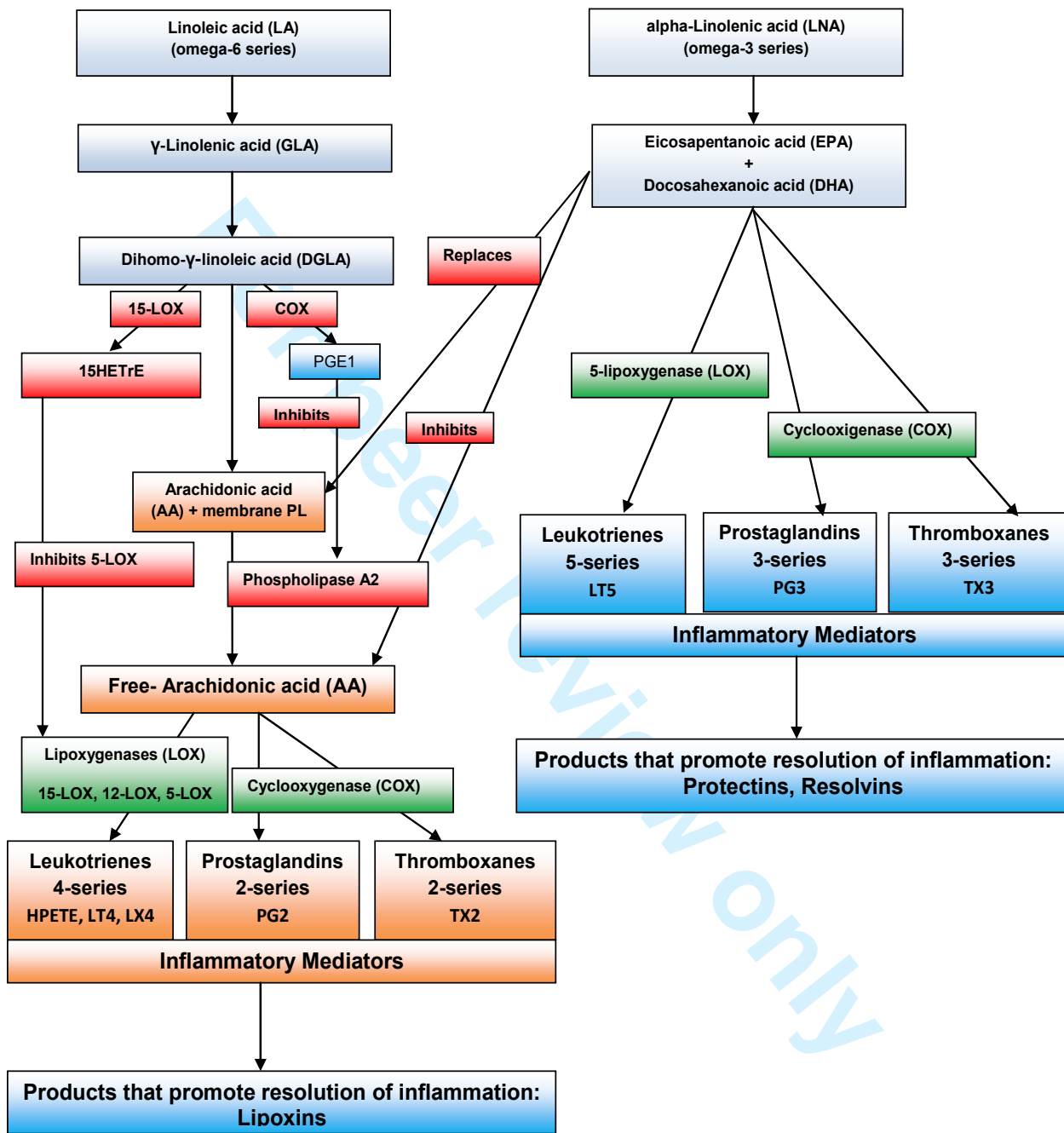
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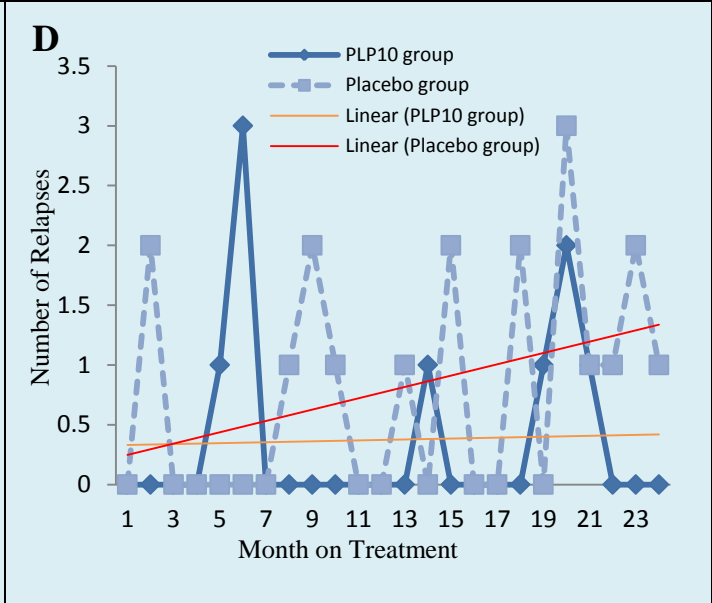
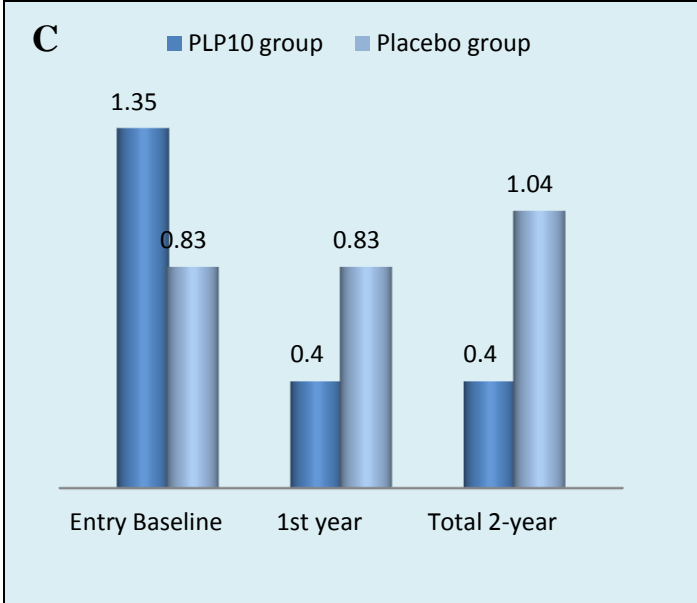
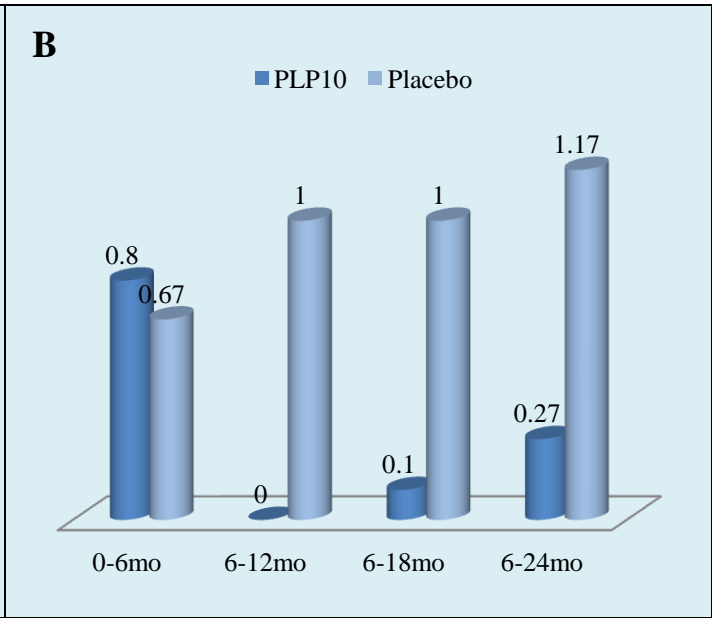
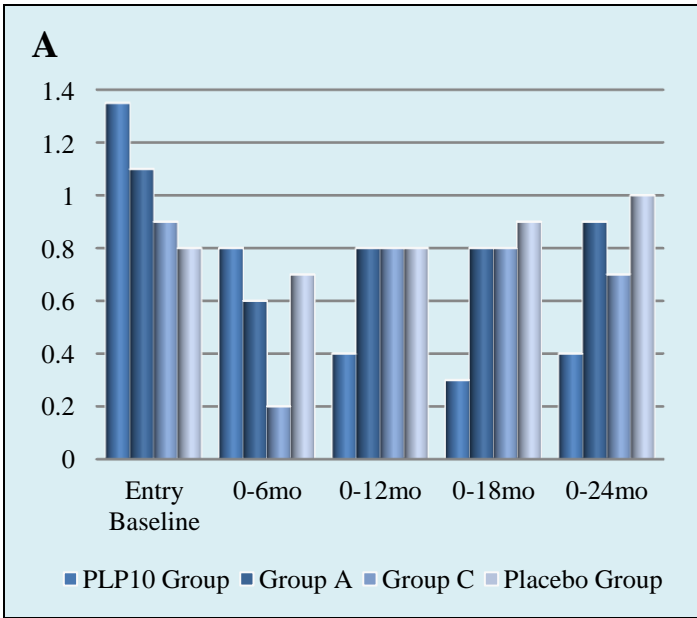


Omega-6 and omega-3 PUFA Consumption through Diet



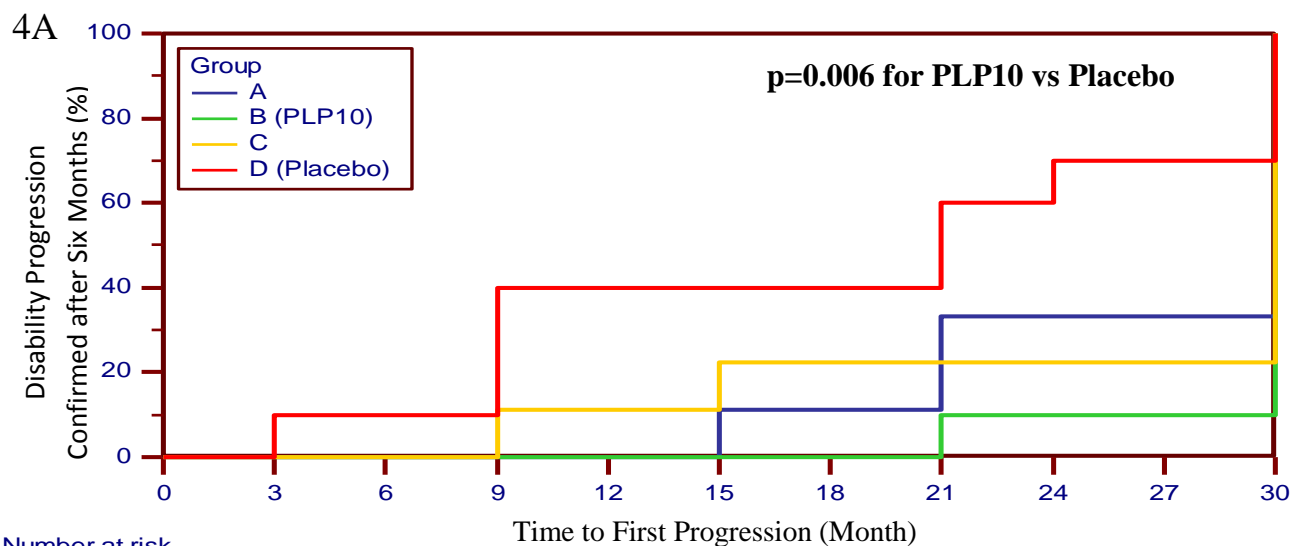
Possible effects on inflammation:
 Reduce IFN- γ production; Reduce IL-2 production; Increase TGF β activity; Increase PGE2 activity; Inhibit arachidonic acid; Reduce TNF levels; RXR- γ and PPAR γ agonist; NF κ B expression; stimulate Nrf2; reduce MMP-2, -3, -9, and -13

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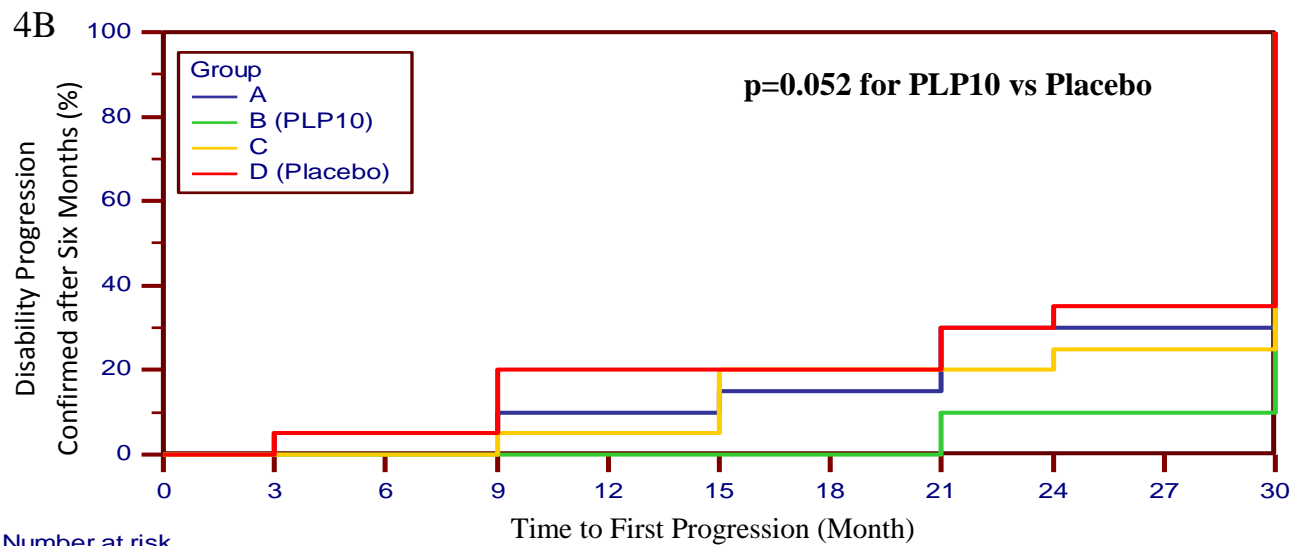


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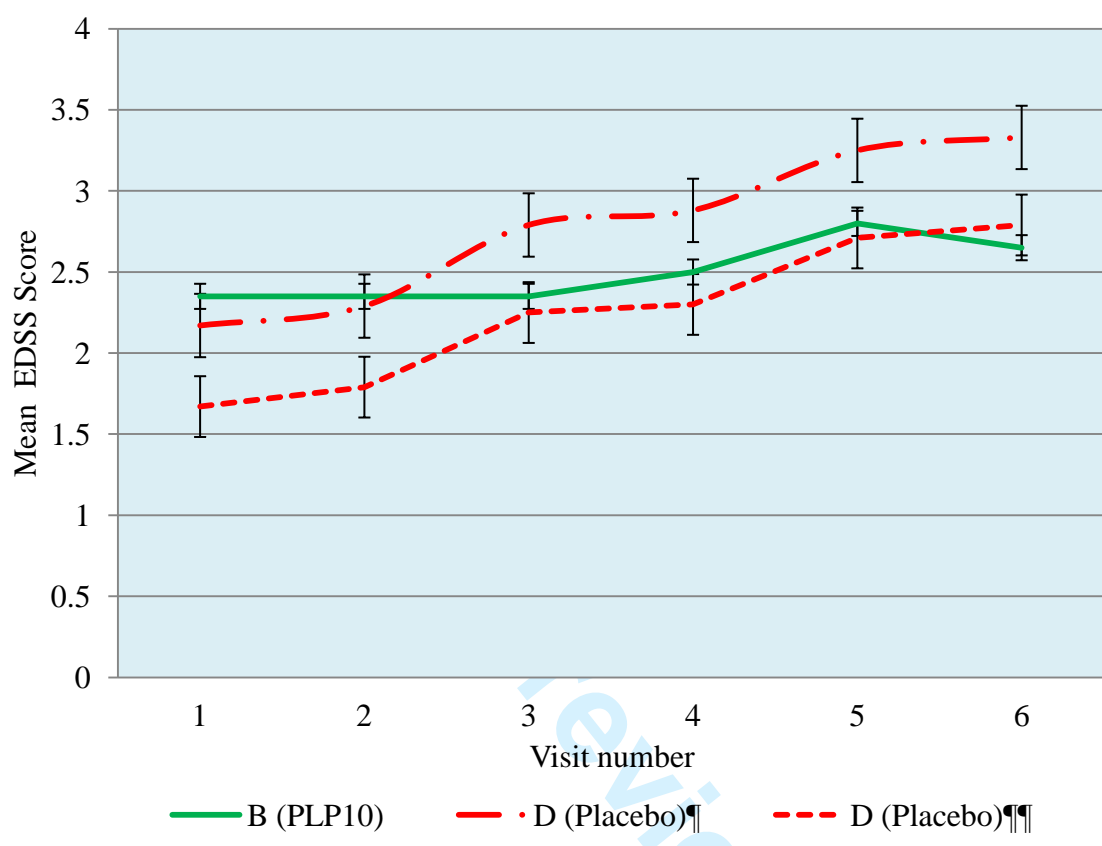


Number at risk	0	3	6	9	12	15	18	21	24	27	30
Group: A	9	9	9	9	9	8	8	6	6	6	6
Group: B (PLP10)	10	10	10	10	10	10	10	9	9	9	9
Group: C	9	9	9	8	8	7	7	7	7	7	7
Group: D (Placebo)	10	9	9	6	6	6	6	4	3	3	3



Number at risk	0	3	6	9	12	15	18	21	24	27	30
Group: A	20	19	19	18	18	17	17	14	14	14	14
Group: B (PLP10)	20	20	20	20	20	20	20	18	18	18	18
Group: C	20	20	20	19	19	16	16	16	15	15	15
Group: D (Placebo)	20	19	19	16	16	16	16	14	13	13	13

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3 **1 Supplementary Information**
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5 **2 Table of Content**
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34 **Supplementary Information Methods 1**

35 **Intervention and study rational.** We are using high dosage of 1:1 omega-3:omega-6
36 polyunsaturated (PUFA) aiming to overpass and normalize global diet regional traditions and
37 habits in relation to abnormal PUFA/saturated fatty acids (SFA)/monounsaturated fatty acids
38 (MUFA) daily ratio consumption irrespective of the quantities consumed; and enough to
39 equilibrate patients' diet in line with the recommended more physiologic omega-3:omega-6
40 ratio of about 1:1-4 wt/wt as reported by Simopoulos, 2002. In addition to correct existing
41 deficiencies, cell membrane abnormalities, specifically of the immunopathological system
42 and blood mononuclear peripheral cells, and high enough for availability and immediate
43 ongoing modulation of the involved pathogenic mechanisms and network of events in MS.
44 The high dosage is also required to overpass the quantity limitations, previously discussed, of
45 diet-consumed PUFAs for cellular incorporation, especially in the central nervous system
46 (CNS) of adults. Additionally, fatty acids (FAs) must first cross the intestinal epithelium
47 before reaching the different tissues, where digestion and absorption constitute further
48 problems in their availability (Carlier, H, 1991). Omega-3 PUFA are used in re-esterified
49 form to eliminate unwanted disturbances, at the sides of action, by other fatty acids and
50 molecules present in crude fish oils but also to increase the bioavailability of the FA since
51 triglycerides have been shown to be associated with much higher bioavailability (Dyerberg et
52 al, 2010). Linoleic (LA) and gamma linolenic acid (GLA) are essential structure molecules
53 and important for any physiological (re)generation of cell membrane. GLA quantity is
54 doubled to LA to ensure high direct production of dihomo-gamma-linolenic acid (DGLA),
55 from GLA when LA cannot be metabolized, due to desaturase deficiency or malfunction.
56 Such a reduced capacity to convert LA to GLA has been associated with aging, diabetes,
57 alcoholism, atopic dermatitis, premenstrual syndrome, rheumatoid arthritis, cancer and
58 cardiovascular diseases (Bolton-Smith et al, 1997; Horrobin, 1990; Leventhal et al, 1993).
59 This is going to result in the increase of DGLA relative to arachidonic acid (AA) with DGLA
60 promoting production of prostaglandin (PG)E1 but also inhibition of phospholipase (PL)A2:
61 two major reasons and rational for their use. If other metabolic problems are involved within
62 the omega-6 series and the normal metabolites are not produced then the eicosapentaenoic
63 acid EPA available in PLP10 will substitute the function of DGLA, as a competitive inhibitor
64 of AA for PLA2. In both cases the pro-inflammatory leucotrienes, prostaglandines of the 2-
65 series (PG2) and thromboxanes of the 2 series including the platelet-activator factor (PAF)
66 will be attenuated. The synthesis of AA from DGLA by $\Delta 5$ desaturase promoted by LA/GLA
67 supplementation is very limited in humans as a result of limited activity of the enzyme
68 (Yang-Yi & Robert, 1998). AA in the body is mostly available through diet. EPA and
69 docosahexaenoic acid (DHA) are both physiologically important and crucial structured
70 molecules able to substitute excess AA and SFA within the cell membranes. EPA will
71 contribute to the inhibition (competitive to AA) of PLA2, joining the co-supplied omega-6
72 PUFA but will also participate in the production of anti-inflammatory leukotrienes,
73 prostaglandins of the 3-series (PG3) and thromboxane (TX3) along with DHA, both found in
74 the PLP10 intervention. Moreover EPA will replace AA of the membrane phospholipids and
75 both omega-3 PUFA will contribute replacing abnormal quantities of SFA and excess AA.
76 DHA is used in 3:1 ratio to EPA to cover any possible inabilities of EPA to be metabolized,
77 high enough to strongly promote high production of the aforementioned anti-inflammatory

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3 78 eicosanoids and cytokines and to be incorporated into CNS cell membranes where DHA
4 79 should be the major PUFA present, replacing other FA, probably saturated and excess of AA.
5 80 EPA, DHA, LA and GLA along with the rest of the other ingredients used (“other” omega-3
6 81 PUFA, SFA and MUFA that are usually found in the cell membranes of healthy people in
7 82 limited quantities) in the intervention regimen are for their availability as minor structural
8 83 constituents of physiological cellular membranes integrity, fluidity and overall function as
9 84 building blocks for myelin repair and/or myelination. Furthermore the PUFA used within the
10 85 cocktail intervention aimed to manipulate all other pathophysiological pathways that are
11 86 reported to be able to: as previously discussed including gene transcription for
12 87 neuroprotection and remyelination. Furthermore, PUFA are used to ensure the integrity of
13 88 blood brain barrier (BBB) and to modulate the gelatinases responsible for the T cell migration
14 89 within the CNS. Three different antioxidant vitamins (vitamin E, mostly as alpha-tocopherol,
15 90 gamma (γ)-tocopherol (vitamin E isoform) and vitamin A) are used in the regimen
16 91 preparation to support the cellular antioxidant defenses but also to protect peroxidation of the
17 92 supplied increased amounts of PUFA. Alpha-tocopherol low-molecular-weight antioxidants
18 93 will contribute to radical scavenging, interfering with gene transcription, protein expression,
19 94 enzyme activity and metal chelation (van Meeteren et al, 2005). Vitamin E (alpha tocopherol)
20 95 and vitamin A are used as antioxidants for the protection of the excess supplemented PUFA,
21 96 with alpha-tocopherol been demonstrated to protect against peroxynitrite-induced oxidative
22 97 damage, as well as able to efficiently detoxify hydroxyl, perhydroxyl and superoxide free
23 98 radicals (the elevated reactive oxygen species (ROS)); each one with different mechanism of
24 99 action, increasing the effect capability (Vatassery et al, 1998b; van Meeteren, 2005). Gamma-
25 100 tocopherol is used in high dosage since its half life is very short compared to alpha-
26 101 tocopherol and has been demonstrated to specifically protect against nitro-radicals.
27 102 Tocopherols can also exert non-antioxidant properties, including modulation of cell signaling
28 103 and immune function, regulation of transcription, and induction of apoptosis as previously
29 104 discussed (van Meeteren et al, 2005).

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39 105 PLP10 is the first preparation ever developed for MS therapy that is composed by the use of
40 106 all different previously discussed PUFA, MUFA, SFA in a cocktail preparation mixed with
41 107 the specific aforementioned antioxidant vitamins that have never been all together used
42 108 before within a specific formulation. The ingredients ratio, quality, structural form and
43 109 mostly the high dosage has never been before tested. Furthermore, the knowledge and
44 110 chronotherapy as well as other unique limitations associated with the individual molecules
45 111 used, have never been accounted, discussed, proposed or reported for any previous
46 112 therapeutic regimen.

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50 113 Through systems medicine therapeutic philosophy, by the use of PLP10, potentially MS
51 114 patients have the opportunity to be treated holistically, by natural source isolated molecules,
52 115 demonstrated as able of affecting and modulating all known pathophysiological,
53 116 immunopathological, habitual, gene related factors; thus the dynamic interconnected complex
54 117 network of events simultaneously. Possibly synergistic effects between PLP10 ingredients are
55 118 also feasible. Moreover we can speculate that treatment efficacy of PLP10, when used as

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3 119 adjunct to existing pharmaceuticals produced by reductionism, can be proven therapeutically
4 120 superior to any available treatment for MS.
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160 **Supplementary Information Methods 2**

161 **Interventions specifications.** The specific omega-3 (re-esterified glycerides) and omega-6
162 (glycerides) raw materials were purchased according to the required interventions' PUFA-
163 fraction specification (molecular structure, quantity/ratio and quality) with vitamin E (alpha-
164 tocopherol) used as antioxidant stabilizer by the supplier. The vitamins and masking aroma
165 were purchased separately. The mixing of fractions to the final required intervention-
166 composition specification was always performed by the same team of scientists under the
167 supervision of the involved medical biochemist and lipidology specialist, under appropriate
168 conditions every six months. Interventions were stored refrigerated in dark until use.

169
170 The ratios of the different ingredients used were as follows: omega-3, EPA to DHA (about 1
171 to 3 wt/wt), omega-6, LA to GLA (about 2 to 1 wt/wt), omega-3 (EPA + DHA) to omega-6
172 (LA + GLA) (about 1 to 1 wt/wt). The total omega-3 (EPA + DHA + "other" omega-3
173 PUFA) used as re-esterified triglycerol (minimum value 60%), diglyceride (about 33%),
174 monoglyceride (about 2%) structural form mixture and about 2% ethyl ester structural form,
175 with no less than 80% re-esterified triglycerol content to be DHA and EPA as a result of
176 PUFA triglycerides re-esterification of fish body oils. The "other" group of omega-3 on the
177 re-esterified glycerols included the 18:3 (alpha-linolenic acid), 18:4 (stearidonic acid), 20:4
178 (eicosatetraenoic acid) and 22:5 (docosapentaenoic acid) PUFA. The fraction of omega-6
179 (LA + GLA) used as triglycerides with no less than 50-65% triglycerol content to be LA and
180 GLA in a ratio of 2 to 1 with 18:1 (oleic acid) 14-20% and 20:1 (eicosenoic acid), 22:1
181 (docosenoic acid), 24:1 (tetracosenic acid) as additional monounsaturated fatty acids and
182 minor quantities of 16:0 (palmitic acid) 4-16%, 18:0 (stearic acid) 2-5% saturated fatty acids
183 from Borage oil source. The vitamins used were vitamin A as beta-carotene, vitamin E
184 (alpha-tocopherol) and pure gamma-tocopherol (vitamine E isoform). Citrus extract was used
185 as masking aroma and pure virgin olive oil as delivery vehicle.

186
187 **The daily intervention formula agent dosages were:**

188 **Intervention formula A** daily dosage: EPA (1650mg) / DHA (4650mg) / GLA (2000mg) /
189 LA (3850mg) / total other omega-3 (600mg) / total monounsaturated fatty acids (MUFA)
190 (18:1 1300mg, 20:1 250mg, 22:1 82mg, 24:1 82mg) + total saturated fatty acids (SFA) (18:0
191 160mg, 16:0 650mg) / vitamin A (0.6mg) / vitamin E (22mg).

192 **Intervention formula B (PLP10)** daily dosage: EPA (1650mg) / DHA (4650mg) / GLA
193 (2000mg) / LA (3850mg) / total other omega-3 (600mg) / total MUFA (18:1 1300mg, 20:1
194 250mg, 22:1 82mg, 24:1 82mg) + total SFA (18:0 160mg, 16:0 650mg) / vitamin A (0.6mg) /
195 vitamin E (22mg) / gamma- tocopherol (γ -tocopherol) (760 mg).

196 **Intervention formula C** daily dosage: γ -tocopherol (760 mg) (in 16137 mg pure virgin olive
197 oil as a vehicle).

198 **Intervention formula D** daily dosage: pure virgin olive oil (16930mg).

199 Citrus aroma was added in each intervention formula to make up a total dosage of 19.5ml of
200 solution per day.

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3 201 The specific omega-3 related fraction, according to specifications required for the
4 202 interventions was prepared and purchased from EPAX AS, Aalesund, Norway; as re-
5 203 esterified glycerides from fish body oils as a source. The specific omega-6 PUFA, MUFA
6 204 and SFA related fraction, according to required specifications, was prepared and purchased
7 205 from Goerlich Pharma International GmbH, Edling, Germany, as triglycerides from Borage
8 206 seed oil (organic, cold pressed) "*Borago officinalis*" as a source. Both omega-3 and omega-6
9 207 fractions were delivered stabilized by the producer (vitamine E (alpha-tocopherol) ~ 4.5 mg/g
10 208 was used as antioxidant).

14 209 Vitamins: vitamin A as beta-carotene (HealthAid Ltd., Middlesex, United Kingdom) and pure
15 210 gamma-tocopherol (Tama Biochemical Co. Ltd., Shinjuku-ku Tokyo, Japan).

17 211 Citrus aroma (Givaudan Schwaiz AG, Dubendorf, Switzerland).

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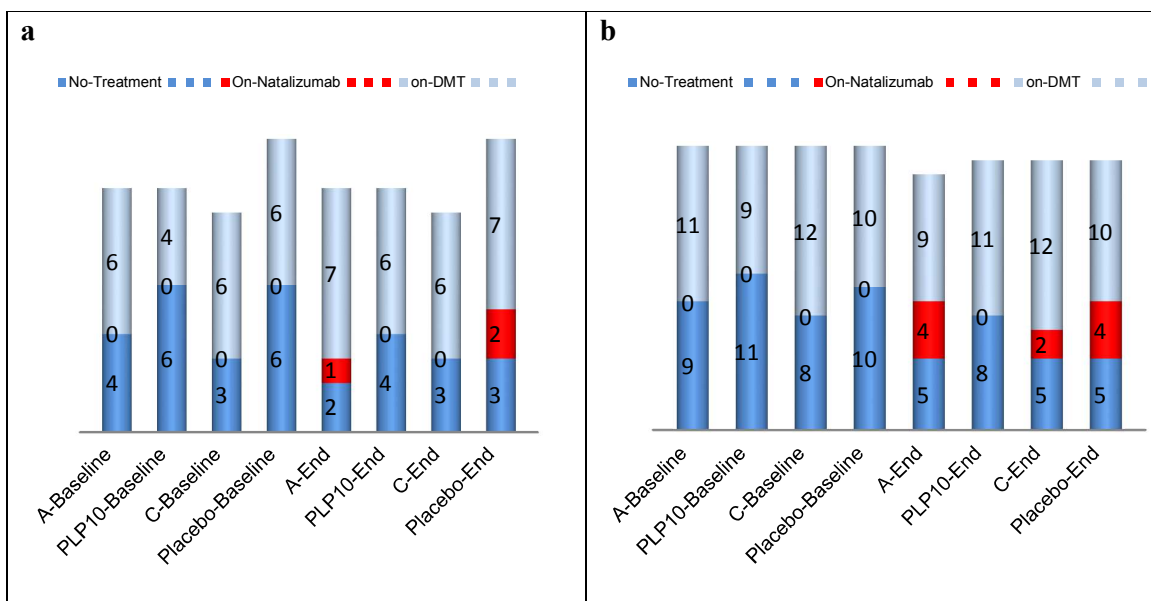
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Supplementary Information Figure 1 | Population on DMT and/or natalizumab. (a) Demonstrates the all-time on-study population per treatment arm that was receiving or not receiving DMT at entry baseline and the same population at the end of the trial (including patients on natalizumab). No statistical significant differences were calculated. The all-time on-study patients per treatment-arm that were receiving DMT at entry baseline were six patients out of ten (60%) within group A, four out of ten (40%) within PLP10 group, six out of nine (66%) within group C and six out of 12 (50%) within placebo. When the study completed, 80% of the patients in group A, 60% in PLP10 group, 66% in group C and 75% of the patients in placebo ended up on treatment. Within group A one out of eight and within placebo two out of nine patients on DMT transferred on natalizumab. (b) Demonstrates the total randomized population per treatment arm that was receiving or not receiving DMT at entry baseline and the same population at the end of the trial without lost to follow (including patients on natalizumab). A total of 61% of group A patients were on DMT at entry baseline and became 72% at the end; for PLP10 group 41% and became 53%; for group C 73% and became 74% and for placebo 53% and became 74% at study completion. At the end: for group A, four out of 13 patients on DMT transferred on natalizumab; for PLP10 group no patient was on natalizumab; for Group C two out of 14 patients on DMT transferred on natalizumab; and for placebo group four out of the 14 patients on DMT transferred on natalizumab. No significant differences measured at entry baseline between the groups.

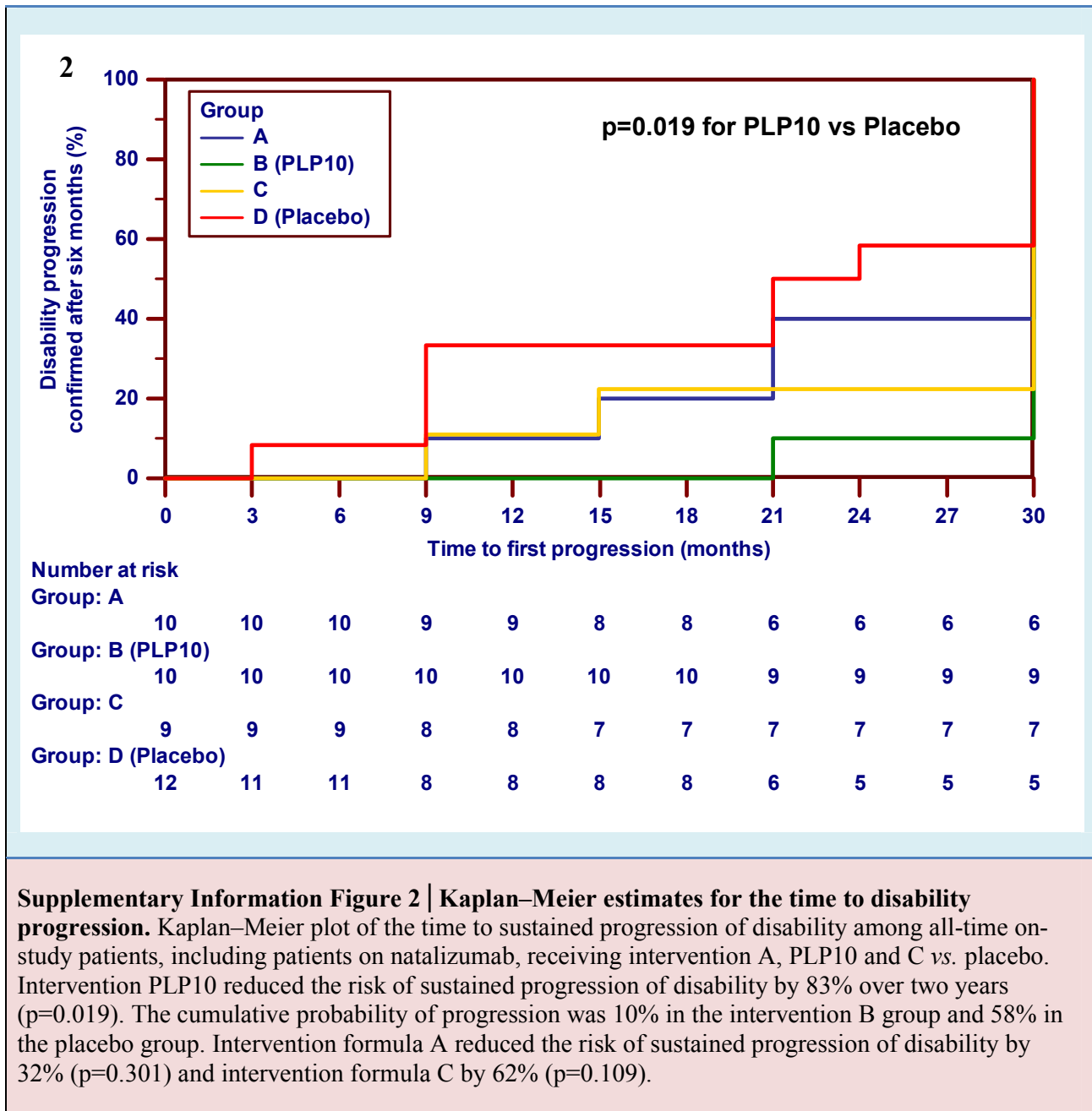
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Checklist of Items for Reporting Trials of Nonpharmacologic Treatments*

Section	Item	Standard CONSORT Description	Extension for Nonpharmacologic Trials	Reported on Page No.
Title and abstract†	1	How participants were allocated to interventions (e.g., “random allocation,” “randomized,” or “randomly assigned”)	In the abstract, description of the experimental treatment, comparator, care providers, centers, and blinding status	1,3,4
Introduction				
Background	2	Scientific background and explanation of rationale		5 to 8
Methods				
Participants†	3	Eligibility criteria for participants and the settings and locations where the data were collected	When applicable, eligibility criteria for centers and those performing the interventions	8, 13,15
Interventions†	4	Precise details of the interventions intended for each group and how and when they were actually administered	Precise details of both the experimental treatment and comparator	9,10,11, Table 1 p.28, Appendix p. 5,6
	4A		Description of the different components of the interventions and, when applicable, descriptions of the procedure for tailoring the interventions to individual participants	10, Table 1 p.28, Appendix p.5
	4B		Details of how the interventions were standardized	9,10, Table 1 p.28, Appendix p.5
	4C		Details of how adherence of care providers with the protocol was assessed or enhanced	9
Objectives	5	Specific objectives and hypotheses		7,8
Outcomes	6	Clearly defined primary and secondary outcome measures and, when applicable, any methods used to enhance the quality of measurements (e.g., multiple observations, training of assessors)		12
Sample size†	7	How sample size was determined and, when applicable, explanation of any interim analyses and stopping rules	When applicable, details of whether and how the clustering by care providers or centers was addressed	14

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5	Randomization– sequence generation†	8	Method used to generate the random allocation sequence, including details of any restriction (e.g., blocking, stratification)	When applicable, how care providers were allocated to each trial group
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8	Allocation concealment	9	Method used to implement the random allocation sequence (e.g., numbered containers or central telephone), clarifying whether the sequence was concealed until interventions were assigned	9
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12	Implementation	10	Who generated the allocation sequence, who enrolled participants, and who assigned participants to their groups	9
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15	Blinding (masking)†	11A	Whether or not participants, those administering the interventions, and those assessing the outcomes were blinded to group assignment	Whether or not those administering co- interventions were blinded to group assignment
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20		11B		If blinded, method of blinding and description of the similarity of interventions†
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22	Statistical methods†	12	Statistical methods used to compare groups for primary outcome(s); methods for additional analyses, such as subgroup analyses and adjusted analyses	When applicable, details of whether and how the clustering by care providers or centers was addressed
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26	Results			
27	Participant flow†	13	Flow of participants through each stage (a diagram is strongly recommended)--- specifically, for each group, report the numbers of participants randomly assigned, receiving intended treatment, completing the study protocol, and analyzed for the primary outcome; describe deviations from study as planned, together with reasons	The number of care providers or centers performing the intervention in each group and the number of patients treated by each care provider or in each center
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35	Implementation of intervention†	New item		Details of the experimental treatment and comparator as they were implemented
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37	Recruitment	14	Dates defining the periods of recruitment and follow-up	10,15,16 Appendix p..5, 11,15
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39	Baseline data†	15	Baseline demographic and clinical characteristics of each group	When applicable, a description of care providers (case volume, qualification, expertise, etc.) and centers (volume) in each group
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Numbers analyzed	16	Number of participants (denominator) in each group included in each analysis and whether analysis was by “intention-to-treat”; state the results in absolute numbers when feasible (e.g., 10/20, not 50%)		15,16
Outcomes and estimation	17	For each primary and secondary outcome, a summary of results for each group and the estimated effect size and its precision (e.g., 95% confidence interval)		15 to 20
Ancillary analyses	18	Address multiplicity by reporting any other analyses performed, including subgroup analyses and adjusted analyses, indicating those prespecified and those exploratory		20
Adverse events	19	All important adverse events or side effects in each intervention group		20
Discussion				
Interpretation†	20	Interpretation of the results, taking into account study hypotheses, sources of potential bias or imprecision, and the dangers associated with multiplicity of analyses and outcomes	In addition, take into account the choice of the comparator, lack of or partial blinding, and unequal expertise of care providers or centers in each group	21
Generalizability†	21	Generalizability (external validity) of the trial findings	Generalizability (external validity) of the trial findings according to the intervention, comparators, patients, and care providers and centers involved in the trial	22
Overall evidence	22	General interpretation of the results in the context of current evidence		22 to 26

*Additions or modifications to the CONSORT checklist. CONSORT = Consolidated Standards of Reporting Trials.

†This item was modified in the 2007 revised version of the CONSORT checklist.



A novel oral nutraceutical formula (PLP10) for the treatment of relapsing remitting multiple sclerosis: a randomized, double-blind, placebo-controlled proof-of-concept clinical trial.

Journal:	<i>BMJ Open</i>
Manuscript ID:	bmjopen-2012-002170.R3
Article Type:	Research
Date Submitted by the Author:	05-Mar-2013
Complete List of Authors:	Pantzaris, Marios; The Cyprus Institute of Neurology and Genetics (CING), Clinic C and PALUPA Medical Ltd Loukaides, George; The Cyprus Institute of Neurology and Genetics (CING), Neurology Clinic and PALUPA Medical Ltd Ntzani, Evangelia; University of Ioannina School of Medicine (UISM), Clinical and Molecular Epidemiology Unit, Department of Hygiene and Epidemiology Patrikios, Ioannis; The Cyprus Institute of Neurology and Genetics (CING), Clinic C and PALUPA Medical Ltd; European University Cyprus, Health Science
Primary Subject Heading:	Nutrition and metabolism
Secondary Subject Heading:	Neurology, Complementary medicine, Pharmacology and therapeutics
Keywords:	Multiple sclerosis < NEUROLOGY, NUTRITION & DIETETICS, Neurophysiology < NEUROLOGY, COMPLEMENTARY MEDICINE, Neurobiology < BASIC SCIENCES, PUBLIC HEALTH

SCHOLARONE™
Manuscripts

1 **A novel oral nutraceutical formula of omega-3 and omega-**
2 **6 fatty acids with vitamins (PLP10) in relapsing remitting**
3 **multiple sclerosis: a randomised, double-blind, placebo-**
4 **controlled proof-of-concept clinical trial**

5 **Marios C. Pantzaris***, **George N. Loukaides**, **Evangelia E. Ntzani**, **Ioannis**
6 **S. Patrikios***

7 * Both M.C.P and I.S.P are the first authors and both are the corresponding authors

8
9 The Cyprus Institute of Neurology and Genetics (CING) 1683 Nicosia, Cyprus Marios C.
10 Pantzaris Senior Consultant Neurologist Neurology Clinic C and PALUPA Medical Ltd., The
11 Cyprus Institute of Neurology and Genetics (CING) 1683 Nicosia, Cyprus George N.
12 Loukaides Clinical Dietitian/Nutritionist Neurology Clinic and PALUPA Medical Ltd.,
13 University of Ioannina School of Medicine (UISM) 45110 Ioannina, Greece Evangelia E.
14 Ntzani Assistant Professor Clinical and Molecular Epidemiology Unit, Department of
15 Hygiene and Epidemiology, PALUPA Medical Ltd at The Cyprus Institute of Neurology and
16 Genetics (CING) 1683 Nicosia, Cyprus Ioannis S. Patrikios Senior Research Scientist,
17 visiting Professor at European University Cyprus Nicosia, Cyprus, Department of Health and
18 Science. Correspondence should be addresses to e-mail: I.Patrikios@euc.ac.cy or
19 pantzari@cing.ac.cy

1
2
3 22 **Correspondence to:**
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5 23 **Ioannis Patrikios**

6
7 24 The Cyprus Institute of Neurology and Genetics (CING)

8
9 25 Neurology Clinic C (PALUPA Medical),

10
11 26 6 International Airport Av.

12
13 27 P.O. Box 23462, 1683 Ayios Dometios. Nicosia, Cyprus

14
15 28 Tel: +357 22 358 600, +357 99 097 856;

16
17 29 i.patrikios@euc.ac.cy

18
19 30 patrikiosioannis@gmail.com
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32 **AND**

33
34 **Marios Pantzaris**

35 The Cyprus Institute of Neurology and Genetics (CING)

36 Neurology Clinic C (PALUPA Medical),

37 6 International Airport Av.

38 P.O. Box 23462, 1683 Ayios Dometios, Nicosia Cyprus

39 Tel: +357 22 358 600; +357 99 677 067

40 pantzari@cing.ac.cy
41
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42 **Keywords:** antioxidant vitamins, polyunsaturated fatty acids, multiple sclerosis, systems
43 medicine, randomised clinical trial.

45 **Word Count: 5915**

1
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3 46 **Abstract**
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5 47 **Objective** To assess whether three novel interventions, formulated based on a systems
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8 48 medicine therapeutic concept, reduced disease activity in patients with relapsing remitting
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10 49 multiple sclerosis who were either treated or not with disease-modifying treatment.
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14 51 **Design** A 30-month randomised, double-blind, placebo-controlled, parallel design, phase II
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17 52 proof-of-concept clinical study.
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21 54 **Settings** Cyprus Institute of Neurology and Genetics (CING)
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26 56 **Participants and Interventions** Eighty subjects were randomised into four groups of twenty.
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28 57 The first intervention (A) was composed of omega-3 and omega-6 polyunsaturated fatty acids
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30 58 at 1:1 wt/wt. Specifically, the omega-3 fatty acids were docosahexaenoic acid (DHA) and
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32 59 eicosapentaenoic acid (EPA) at 3:1 wt/wt, and the omega-6 fatty acids were linoleic acid
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34 60 (LA) and gamma (γ)-linolenic acid (GLA) at 2:1 wt/wt. This intervention also included minor
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36 61 quantities of other specific polyunsaturated, monounsaturated and saturated fatty acids as
37
38 62 well as vitamin A and vitamin E (alpha-tocopherol). The second intervention (B, PLP10) was
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40 63 a combination of A and γ -tocopherol. The third intervention (C) was γ -tocopherol alone. A
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42 64 fourth group of 20 received placebo. The interventions were administered per os (by mouth)
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44 65 once daily.
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50 67 **Main outcome measures** The primary endpoint was the annualised relapse rate (ARR) of the
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52 68 three interventions versus the placebo at two years. The secondary end point was the time to
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54 69 confirmed disability progression at two years.
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3 71 **Results** A total of 41 (51%) patients completed the 30-month trial. Overall, for the per-
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5 72 protocol analysis of the two-year primary end point, 8 relapses were recorded in the PLP10
6
7 73 group (n=10) (0.40 ARR) versus 25 relapses in the placebo group (n=12) (1.04 ARR),
8
9 74 representing a 64% adjusted relative rate reduction for the PLP10 group (RRR 0.36, 95% CI
10
11 75 0.15 to 0.87, p=0.024). In a subgroup analysis that excluded patients on monoclonal antibody
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13 76 (natalizumab) treatment, the observed adjusted RRR became stronger (72%) over the two
14
15 77 years (RRR 0.28, 95% CI 0.10 to 0.79, p=0.016). The per-protocol analysis for the secondary
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17 78 outcome at two years, the time to disability progression, was significantly longer only for
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19 79 PLP10. The cumulative probability of disability progression at two years was 10% in the
20
21 80 PLP10 group and 58% in the placebo group (unadjusted log-rank p=0.019). In a subgroup
22
23 81 analysis that excluded patients on natalizumab, the cumulative probability of progression was
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25 82 10% for the 10 patients in the PLP10 group and 70% for the 12 patients in the placebo group,
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27 83 representing a relative 86% decrease in the risk of the sustained progression of disability in
28
29 84 the PLP10 group (unadjusted log-rank p=0.006; adjusted hazard ratio, 0.11; 95% CI 0.01 to
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31 85 0.97, p=0.047). No adverse events were reported. Interventions A (10 patients) and C (9
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33 86 patients) showed no significant efficacy.
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88 **Conclusions** In this small proof-of-concept, randomised, double-blind clinical trial; the
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90 PLP10 treatment significantly reduced the ARR and the risk of sustained disability
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92 progression without any reported serious adverse events. Larger studies are needed to further
93
94 assess the safety and efficacy of PLP10.
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93 **Trial registration** International Standard Randomised Controlled Trial, number
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95 ISRCTN87818535.

96 Introduction

97 Multiple sclerosis (MS) is a complex multifactorial disease that results from the interplay
98 between environmental factors and a susceptible genetic background.¹⁻³ Together, these
99 factors trigger a cascade of events involving the engagement of the immune system,
100 inflammatory injury of myelin, axons and glia, functional recovery and structural repair,
101 gliosis, and neurodegeneration.⁴ The mechanisms involved include immune-mediated
102 inflammation, oxidative stress and excitotoxicity, all of which contribute to oligodendrocyte
103 and neuronal damage and even cell death, hence promoting disease progression.⁵⁻⁹ The
104 increasing prevalence of MS combined with the partial efficacy and side effects of the
105 existing treatments have urged the development of new, innovative, more effective, safe, and
106 preventive treatment strategies.

107
108 Recent research has shown that multiple variables dynamically interact and many different
109 complex interrelated processes are simultaneously orchestrated for MS pathogenesis. The
110 uniqueness of systems medicine (SM) is the recognition that different specific complex
111 factors are important in disease management and that these factors need to be incorporated in
112 some meaningful way for treatment selection and delivery.¹⁰ The primary challenge of a
113 systems scientific approach is the elucidation of how these multiple variables dynamically
114 interact and how this understanding can be applied to affect the system and achieve a
115 desirable end.^{10 11} One approach towards that end might be the simultaneous intervention in
116 multiple involved pathways using a combination of different active ingredients that could
117 exert a synergistic effect and provide a comprehensive, sustainable treatment effect
118 (Supplementary Information Methods 1).

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3 120 The polyunsaturated fatty acid (PUFA) composition of membrane phospholipids plays an
4
5 121 important role in immune- and non-immune-related inflammation. PUFA and antioxidant
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7 122 deficiencies along with a decreased cellular antioxidant defence mechanisms have been
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10 123 reported in MS patients.¹²⁻¹⁵ The cause of PUFA deficiencies is not entirely clear and may
11
12 124 involve metabolic and nutritional alterations.¹²

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16 126 Increased or uncontrolled inflammation contributes to several different acute and chronic
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18 127 diseases, and it is characterised by the production of inflammatory cytokines, arachidonic
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20 128 acid (AA)-derived eicosanoids (prostaglandins [PGs], thromboxanes [TXs], leukotrienes
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22 129 [LTs], and other oxidised derivatives), and other inflammatory agents such as reactive
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25 130 oxygen species (ROS), nitric oxide (NO), and adhesion molecules (Fig 2).¹⁶ During
26
27 131 inflammation, glutamate homeostasis is altered by the release of increased quantities of
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29 132 glutamate by activated immune cells, which can result in the over-activation of glutamate
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31 133 receptors and, in turn, excitotoxic oligodendroglial death.^{7 17} Among others, membrane-
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33 134 related pathology, immune-mediated inflammation, oxidative stress, and excitotoxicity
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35 135 provide potentially useful combined targets for intervention in MS.

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41 137 *In vitro* and *in vivo* studies have demonstrated that dietary eicosapentaenoic acid (EPA),
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43 138 docosahexaenoic acid (DHA), linoleic acid (LA), and gamma (γ)-linolenic acid (GLA) can be
44
45 139 implicated and modulate almost all known complex networks of events and pathways in MS
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47 140 pathophysiology. The brain membrane fatty acid composition can be modified with dietary
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49 141 supplementation, but the process has been shown to be age dependent (taking much longer in
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51 142 adults versus developing brains) and possibly dependent on the quantity of the
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53 143 dietary/supplemented PUFAs.¹⁸ Both human and animal studies proved that diets high in
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55 144 DHA and EPA can increase the proportion of these PUFAs in the membranes of
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3 145 inflammatory cells and also reduce the levels of AA, a stress-related biomarker.^{16 19} The anti-
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5 146 inflammatory properties of omega-3PUFAs include the production of PGs and TXs of the 3-
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7 147 series and LTs of the 5-series (Fig 2).^{18 20} Resolvins and protectins are biosynthesised from
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9 148 omega-3 fatty acids via cyclooxygenase-2/lipoxygenase (COX-2/LOX) pathways, and they
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11 149 promote the control of inflammation in neural tissues (Fig 2).²¹⁻²⁵ T-cell proliferation in acute
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14 150 and chronic inflammation can also be reduced by supplementation with either omega-6 or
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16 151 omega-3 PUFAs.²⁶ Furthermore, vitamin E is an important antioxidant that can interrupt the
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18 152 propagation of free radical chain reactions.²⁷ Specifically, vitamin E (alpha-tocopherol, an
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20 153 isoform of vitamin E) efficiently detoxifies hydroxyl, perhydroxyl and superoxide free
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22 154 radicals, whereas γ -tocopherol (another isoform of vitamin E) appears to be more efficiently
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24 155 implicated in trapping NO radicals.^{28 29} In addition, alpha-tocopherol exerts non-antioxidant
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26 156 properties, including the modulation of cell signalling and immune functions, regulation of
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28 157 transcription, and induction of apoptosis.³⁰
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34 159 Moreover, omega-3 fatty acid electrophilic derivatives formed by COX-2 in activated
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36 160 macrophages can stimulate the nuclear respiratory factor (Nrf2), which induces the
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38 161 transcription of neuroprotective and antioxidant-related genes and can activate the
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40 162 peroxisome proliferator-activated receptor (PPAR) γ for an anti-inflammatory response.³¹⁻³⁴ In
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42 163 animal studies, EPA and DHA proved to be endogenous ligands of RXRs, with positive
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44 164 effects on neurogenesis.³⁵ Additionally, in 2008, Salvati and coworkers reported evidence of
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46 165 accelerated myelination in DHA- and EPA-treated animals.³⁶ Moreover, DHA and EPA have
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48 166 been reported to significantly decrease the levels of metalloproteinases (MMP) -2, -3, -9, and
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50 167 -13, which have a significant role in the migration of lymphocytes into the central nervous
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52 168 system (CNS) by inducing the disruption of the blood brain barrier (BBB), an important step
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54 169 in the formation of MS lesions.³⁷⁻⁴³
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5 171 Based on the above observations, specific PUFAs and antioxidant vitamins fulfil the criterion
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7 172 of biologic plausibility and have the potential to diminish the severity and activity of MS
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9 173 symptoms, potentially even promoting recovery (remyelination).^{12 44}

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14 175 We report here a randomised phase II, single-centre, double-blind, placebo-controlled, proof-
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16 176 of-concept clinical trial evaluating the therapeutic ability of a nutraceutical formula (with
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18 177 PLP10 representing the complete composition of the formulation) and of two other
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20 178 interventions (A and C) consisting of PLP10-constituent partial fractions containing
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22 179 ingredients for the aforementioned substance categories on relapsing remitting (RR) MS
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24 180 patients.

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31 32 183 **Methods**

33 34 184 **Patients**

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37 185 The eligibility criteria were an age of 18 to 65; a diagnosis of RRMS according to the
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39 186 McDonald criteria; a score of 0.0 to 5.5 on the Expanded Disability Status Scale (EDSS), a
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41 187 rating that ranges from 0 to 10, with higher scores indicating more severe disability; MRI
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43 188 showing lesions consistent with MS; at least one documented clinical relapse; and either
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45 189 receiving or not a disease-modifying treatment (DMT) within the 24 month period before
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47 190 enrolment in the study. Patients were excluded because of a recent (<30 days) relapse, prior
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49 191 immunosuppressant or monoclonal antibody therapy, pregnancy or nursing, other severe
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51 192 disease compromising organ function, progressive MS, history of recent drug or alcohol
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53 193 abuse, use of any additional food supplements, vitamins, or any form of PUFA, and history of
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55 194 severe allergic or anaphylactic reactions or known specific nutritional hypersensitivity. No
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3 195 monitoring or limitations on the patients' daily dietary habits were considered because the
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5 196 high quantities of the ingredients within the formula could not be significantly affected by
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7 197 any particular dietary pattern.
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11 199 The study was conducted in accordance with the standards of the International Conference of
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14 200 Harmonisation Guidelines for Good Clinical Practice. The protocol was developed by the
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16 201 investigators, approved by the Cyprus National Bioethics Committee, and overseen by an
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18 202 independent safety-monitoring committee evaluating the safety and over-all benefit-risk
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20 203 profiles. The adherence of the care providers with the protocol was assessed by an external
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23 204 committee assigned by the funder of the project through reviews of case report forms. All
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25 205 patients gave written informed consent at the time of enrolment.
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28 29 207 **Randomisation and masking**

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31 208 Patients were randomly assigned to four intervention groups in a 1:1:1:1 ratio stratified by
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33 209 gender (women to men, 3:1). Randomisation was facilitated by a lottery-type pool of
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35 210 numbered balls. Patients were randomly assigned to the treatments in blocks of four by
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37 211 flipping a coin as follows: for the first two drawn balls, heads stratified them to the groups
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39 212 A/B and tails stratified them to the groups C/D. The other two balls were stratified
40
41 213 accordingly. A second toss of the coin assigned the two patients to group A (head)/B (tail) or
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43 214 to group C (head)/D (tail). The randomisation scheme was generated, performed and securely
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45 215 stored by the Helix Incubator Organization of Nicosia University (HIONU).
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51 217 The interventions had identical appearances and smells and were kept in dark bottles (15
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53 218 daily-dose portions/bottle) under a nitrogen bed and labelled by HIONU with code numbers,
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55 219 blinded for both the patients and investigators. Study data were collected by the investigators
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3 220 and saved by HIONU, which also held the blinded codes for the study. All study personnel
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5 221 involved in the conduct of the study were blinded throughout the study. The
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7 222 treating/examining physician, other investigators, pharmacist, neuroradiologist and patients
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9 223 were masked to the treatment allocation.
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14 225 **Procedures and end points**

16 226 The specific omega-3 and omega-6 raw materials were purchased according to the required
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18 227 interventions' PUFA-fraction specification (molecular structure, quantity/ratio and quality)
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20 228 with vitamin E (alpha-tocopherol) used as antioxidant stabiliser by the supplier. The vitamins
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22 229 and masking aroma were purchased separately. The mixing of the fractions to the final
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24 230 required intervention-composition specification was always performed by the same team of
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26 231 scientists under the supervision of the involved medical biochemist and lipidology specialist
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28 232 and under appropriate conditions every six months. The interventions were refrigerated in the
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30 233 dark until use. See Table 1 and Supplementary Information Methods 1 and 2 for a detailed
31
32 234 description of the interventions.
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38 236 The participants were randomly assigned to receive the following: group A, a daily dose of a
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40 237 19.5 ml mixture of EPA (1,650 mg) / DHA (4,650 mg) / GLA (2,000 mg) / LA (3,850 mg) /
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42 238 total other omega-3 (600 mg) / total MUFA (1,714 mg) + total SFA (18:0 160 mg and 16:0
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44 239 650 mg) / vitamin A (0.6 mg) / vitamin E (22 mg) plus citrus-aroma (intervention A); group
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46 240 B (PLP10), a daily dose of a 19.5 ml mixture of EPA (1,650 mg) / DHA (4,650 mg) / GLA
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48 241 (2,000 mg) / LA (3,850 mg) / total other omega-3 (600 mg) / total MUFA (1,714 mg) + total
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50 242 SFA (18:0 160 mg and 16:0 650 mg) / vitamin A (0.6 mg) / vitamin E (22 mg) plus pure γ -
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52 243 tocopherol (760 mg) plus citrus-aroma (intervention B); group C, a daily dose of a 19.5 ml
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54 244 mixture of pure γ -tocopherol (760 mg) dispersed in pure virgin olive oil (16,137 mg) plus
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3 245 citrus-aroma (intervention C); and group D (placebo), a daily dose of a 19.5 ml mixture of
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5 246 pure virgin olive oil (16,930 mg) plus citrus-aroma (intervention D) (Table 1). The
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7 247 pharmacist of the institution was responsible for the appropriate storage and handling of the
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9 248 interventions for the individual participants. The interventions were taken orally once daily
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11 249 30 minutes before dinner using a dosage-calibrated cup for 30 months. The ingredients, ratio
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13 250 and dose were selected based on their biophysical interrelationship with the total known
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15 251 multiple MS causative factors, their biochemical importance and the role they were expected
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17 252 to play in the normalisation and treatment of the involved complex network of events in the
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19 253 disease pathophysiology. Moreover, the high intervention dosage was selected with the aim
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21 254 of optimising the body composition of omega-3 to omega-6 PUFAs to a 1:1 wt/wt ratio
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23 255 irrespective of dietary habits and geographical origin.
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30 257 The period from July 1st 2007 (enrolment) to December 31st 2007 was used as the
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32 258 normalisation period. This six-month normalisation period would allow the interventions to
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34 259 exert their beneficial effect as oral PUFAs need four to six months to achieve pivotal action
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36 260 on immune and neural cells, correction of antioxidant deficiencies and body PUFA
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38 261 redistribution, and an optimal normalisation of the EPA and DHA ratio.⁴⁵⁻⁴⁷ The study was
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40 262 completed on December 31st 2009 (30 months), and the recording of relapses continued until
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42 263 December 31st 2010 (42 months). Overall, the study included a “normalisation period” (July
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44 264 1st 2007 to Dec 31st 2007), an “on treatment” period (Jan 1st 2008, the baseline, to Dec 31st
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46 265 2009) and a 12-month “post-study monitoring period” (Jan 1st 2010 to Dec 31st 2010).
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52 267 Depending on their clinical status and in accordance with common practice, the participants
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54 268 continued receiving their indicated regular treatment, with persistent evaluation for any side-
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56 269 effects and adverse events. Clinical assessment visits were scheduled at baseline and 3, 9, 15,
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3 270 21 and 24 months on-treatment. The patients were also clinically examined by the treating
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5 271 neurologist within 48 hours after the onset of new or recurrent neurologic symptoms.
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9 273 The primary end point was the ARR at two years. A relapse was defined as new or recurrent
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11 274 neurologic symptoms not associated with fever or infection that lasted for at least 24 hours
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13 275 and was accompanied by new neurologic signs. Relapses were treated with methyl-
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15 276 prednisolone at a dose of 1 g intravenous per day for three days, followed by prednisone
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17 277 orally at a dose of 1 mg/kg of weight per day on a tapering scheme for three weeks. The
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19 278 secondary end point at two years was the time to disability progression, defined as an
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21 279 increase of 1.0 or more on the EDSS and confirmed after six months. Progression could not
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23 280 be confirmed during a relapse, and the final EDSS score was confirmed six months after the
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25 281 end of the study. A post-hoc analysis was performed to assess the proportion of patients free
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27 282 from new or enlarging T2 lesions on brain MRI scans at the end of the study for the per-
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29 283 protocol participants of the group receiving the most effective intervention versus placebo.
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31 284 This comparison was made versus the available archival MRI scans up to three months before
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33 285 the enrolment date. The MRI scans were performed and blindly analysed at an MRI
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35 286 evaluation centre. The patients were monitored for an additional 12 months after completion
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37 287 of the trial, and relapses were recorded. The patients were strongly encouraged to remain in
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39 288 the study for follow-up assessments even if they had discontinued the study drug.
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47 290 Blood samples were collected from all randomised patients at the time of enrolment, at every
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49 291 scheduled clinical assessment and during relapses. To evaluate the compliance, the fatty acid
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51 292 composition of the patients' red blood cell membranes was determined by gas
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53 293 chromatography, according to a standard protocol. The fatty acid analyses were performed
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55 294 after study termination and thus did not influence the blinding. Safety measures were
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3 295 assessed from the time of enrolment until 12 months following the study completion.
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5 296 Haematological and biochemical tests were performed at enrolment and every 12 months,
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7 297 including a full blood count, renal and liver function tests, and protein, cholesterol,
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9 298 triglyceride, glucose and electrolyte levels.
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14 300 The involved neurologist was experienced with more than 20 years in practice. He was
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16 301 trained to standardise the EDSS scoring procedures, examined the patients, made all medical
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18 302 decisions, determined the EDSS score and reviewed the adverse effects or side effects. The
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20 303 medical biochemist, who was a specialist in lipidology and immunology, and the registered
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22 304 clinical dietitian were both members of the investigative team with more than 25 years of
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24 305 experience in practice. The patients were able to contact the involved neurologist at any time
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26 306 if there was any adverse event, side effect or allergic reaction. The study drug was not
27
28 307 expected to have any clinical or laboratory adverse effects different from those of the placebo
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30 308 that could disturb the double-blind nature of the trial. Therefore, the study neurologist
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32 309 functioned as both the treating and evaluating physician.
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38 311 The whole procedure followed the clinical trial guidelines as required by the USA Food and
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40 312 Drug Administration, European Medicines Agency, and the Committee for Medicinal
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42 313 Products for Human Use.⁴⁸
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45 315 **Statistical analysis**

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48 316 Power calculations could not be performed before the study because of the lack of
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50 317 information from previous studies on the potential effect sizes. In 2005, the prevalence of MS
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52 318 in Cyprus (600,000 population) was 120/100,000. Based on the aforementioned MS patient
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54 319 numbers in our country and the reference centre, the CING, we were able to enrol 20% of the
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3 320 total RRMS patients eligible for treatment. The sample size was strictly based on the
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5 321 subjects' availability and the novelty of the assessed intervention.
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9 323 The baseline characteristics were compared across all intervention groups by ANOVA or the
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11 324 Kruskal-Wallis rank test for continuous variables and by mean Fisher's exact test for
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13 325 categorical variables, as appropriate.
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17 327 For the primary outcome, the ARR was analysed in a pair-wise fashion for the active
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19 328 interventions compared with the placebo using negative binomial regression models adjusted
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21 329 for the number of relapses within two years, the EDSS score at baseline and DMT. The
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23 330 relapse rate was calculated as the total number of relapses divided by the total number of
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25 331 patient-years followed for each treatment group. ARR differences were also calculated
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27 332 among all comparable parameters and reported as the per-cent difference.
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31 334 For the secondary end-point, the time to disability progression, Kaplan–Meier curves were
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33 335 constructed. The progression of disability and time thereof were compared in a pair-wise
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35 336 fashion for the active interventions versus placebo by the log-rank test in the main analysis
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37 337 and by the Cox proportional-hazards models with adjustments for the baseline EDSS score,
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39 338 age and DMT in the supportive analysis. Multivariate models considered all variables with P
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41 339 <0.1 in the univariate models. There was no overt violation of the proportionality assumption.
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45 341 Both per-protocol and intention to treat (ITT) analyses were performed for different sets of
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47 342 research questions to be answered, and both are reported. Missing data of the five patients
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49 343 lost to follow-up were imputed by the last-observation-carried-forward (LOCF) approach.
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51 344 Due to the proof-of-concept design of the study, the considerable non-adherence rate (49%)
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3 345 and the resulting interpretation issues regarding the ITT analysis, the per-protocol analysis
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5 346 was considered to be the more informative and appropriate method to answer the research
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7 347 questions addressing the efficacy of the interventions when the subjects continuously
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10 348 followed the protocol. All statistical analyses were well defined a priori. All analyses were
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12 349 performed with STATA SE 10.0 (College Station, TX, USA). P-values were two-tailed.
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351 **Role of the funding source**

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18 352 The funders had no role in the study design, data collection and analysis, decision to publish,
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20 353 or preparation of the manuscript. All members of the writing group had full access to all
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22 354 study data, contributed to its interpretation and prepared, reviewed, and approved the
23
24 355 manuscript for submission. All authors had the final responsibility for the decision to submit
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26 356 the paper for publication.
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359 **Results**

360 **Study population**

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39 361 Among the 80 patients, 20 patients were randomly assigned to each of the three groups to
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41 362 receive the interventions, and 20 to receive the placebo (Fig 1). The baseline characteristics
42
43 363 of both the ITT and per-protocol populations were similar across the groups (Table 2A and
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45 364 2B). All patients who dropped out completed the follow-up until the study completion and
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47 365 were included in the ITT analyses (Table 4). Five patients were lost to follow-up before their
48
49 366 first scheduled visit. Two other patients who dropped out before their first scheduled visit
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51 367 progressed to secondary progressive MS. Fifteen patients dropped out without successfully
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53 368 completing the “normalisation” period, including five pregnancies. Another 17 patients
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55 369 dropped out early after the entry baseline. Seven patients who dropped out were given
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3 370 monoclonal antibody treatment (natalizumab). Overall, a total of 41 (51%) patients
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5 371 completed the 42-month study, one patient from group A and two from the placebo group
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7 372 transferred to natalizumab, and 39 (49%) patients either withdrew (dropped out) or were lost
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9 373 to follow-up. The reasons for discontinuation are listed in Figure 2.
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14 375 **Efficacy**

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16 376 **Relapses**

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18 377 As a proof-of-concept trial, we primarily needed to answer whether the interventions were
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20 378 effective for those patients who adhered to the assigned treatment, which was the per-
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22 379 protocol analysis.⁴⁹ For methodological comprehensiveness, we also performed the ITT
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24 380 analysis as a secondary analysis to answer different questions that were complementary to
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26 381 our core hypothesis, such as what happened to all MS patients who were placed on the
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28 382 interventions (the effect of assignment).⁴⁹
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34 384 In the per-protocol analysis, during the first year of the treatment, the ARR was 0.80, 0.40,
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36 385 0.78 and 0.83 for the four intervention groups, respectively. During the second year, the ARR
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38 386 was 0.90, 0.40, 0.67 and 1.25 for the four intervention groups, respectively. Overall, for the
39
40 387 two-year primary end point, 8 relapses were recorded for the 10 patients in the PLP10 group
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42 388 (0.40 ARR) versus 25 relapses for the 12 patients on the placebo (1.04 ARR), a 64% adjusted
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44 389 relative rate reduction (RRR) for the PLP10 group (RRR 0.36, 95% confidence interval [CI]
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46 390 0.15 to 0.87, p=0.024) (Tables 3A, 5 and Fig 3A and 3C). After excluding patients on
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48 391 monoclonal antibody (natalizumab) treatment, the observed adjusted RRR became stronger
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50 392 (72%) over the two years (RRR 0.28, 95% CI 0.10 to 0.79, p=0.016, Tables 3B and 5). Pair-
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52 393 wise comparisons for the other two groups against the placebo did not yield significant results
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54 394 (Tables 3A, 3B). The proportion of patients with ≤ 1 relapse for the two years on-study was
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3 395 higher in the PLP10 group than in the placebo group (90% versus 42%, $p=0.030$, Table 5).
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5 396 Seeking to further investigate the observed difference, we compared the relapse rate during
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7 397 the 24 months before the entry into the study to the 24 months on-treatment for each
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9 398 intervention group. We observed a significant relative reduction in the ARR (70%) only in
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11 399 the PLP10 group (RRR 0.30; 95% CI 0.14 to 0.65, $p=0.003$, Table 3A); within-group
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13 400 comparisons for the ARR reduction of the three other groups were not significant and
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15 401 remained not significant when the natalizumab-treated patients were further excluded from
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17 402 the analysis. The effect of PLP10 through time at different time-windows versus placebo for
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19 403 all-time on-study patients is shown in Figures 3A to 3D. Although the ARR analysis within
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21 404 time-windows was not an assigned endpoint, it could help with the process of evaluating
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23 405 parallel information, such as the efficacy profile through time. PLP10 reached its maximum
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25 406 effect within one year on-treatment (counted from the entry baseline) and remained stable
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27 407 afterwards at an ARR of 0.4 with some free-relapse time-windows. Figure 3D demonstrates
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29 408 the dispersion of relapses throughout the 2-year period of all-time on-study (excluding
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31 409 patients on natalizumab) for PLP10 ($n=10$) versus placebo ($n=10$). The placebo group, in line
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33 410 with the existing knowledge of how the relapse history works in relation to future relapses in
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35 411 MS patients (contagion phenomenon), showed the expected trend of increased relapse
36
37 412 incidences.⁵⁰ The same phenomenon was true for groups A and C. Finally, during the 12
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39 413 month post-study extended period, the on-study patients who received PLP10 showed a
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41 414 persistent benefit in the ARR compared with the placebo (six relapses for the 10 subjects
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43 415 within the PLP10 group, 0.6 ARR versus 19 for the 12 subjects within the placebo group,
44
45 416 1.58 ARR), indicating a statistically significant 62% adjusted relative rate reduction in the
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47 417 ARR for the PLP10 group (RRR 0.38, 95% CI 0.12 to 0.99, $p=0.046$).
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3 419 Regarding the ITT analysis, the relapses of the drop-out patients are reported in Table 4A. As
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5 420 expected, no statistically significant differences in the ARR were calculated for the
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7 421 comparison of any group versus placebo for the 24 months on-treatment (Table 4B). The ITT
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9 422 population on DMT and/or on natalizumab is shown within the Supplementary Information
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11 423 Fig 1. Interestingly, despite the high non-adherence rate, there was a statistically significant
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13 424 difference for the comparison of the ARR in the 24 months before entry baseline with the 24
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15 425 months on-treatment for the PLP10 group (RRR 0.45, 95% CI 0.26 to 0.78, $p=0.005$).
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20 21 427 **Disability progression**

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23 428 In the per-protocol analysis, at two years, the time to disability progression was significantly
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25 429 longer only with PLP10. The cumulative probability of disability progression was 10% in the
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27 430 PLP10 group and 58% in the placebo group ($p=0.019$) (Supplementary Information Fig 2).

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29 431 After excluding the patients on natalizumab, there was again a statistically significant
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31 432 difference between the PLP10 and placebo groups for the same analysis ($p=0.006$) (Fig 4A).

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33 433 At two years, the cumulative probability of disability progression was 10% in the PLP10
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35 434 group and 70% in the placebo group, which represents a decrease of 60 percentage points or a
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37 435 relative 86% decrease in the risk of the sustained progression of disability within the PLP10
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39 436 group (adjusted hazard ratio, 0.11; 95% CI 0.01 to 0.97, $p=0.047$). One versus seven out of
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41 437 ten patients progressed to confirmed disability in the PLP10 and placebo groups, respectively,
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43 438 when patients on natalizumab were excluded. No statistically significant difference was
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45 439 observed for any comparison of the other two groups with the placebo group (Fig 4A and
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47 440 Supplementary Information Fig 2).
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54 442 In the ITT analysis, at two years, the cumulative probability of progression was 10% in the
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56 443 PLP10 group and 35% in the placebo group ($p=0.052$, a trend for an effect), which represents

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3 444 a decrease of 25 percentage points or a relative 71% decrease for the PLP10 group with
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5 445 respect to the risk of sustained progression of disability (adjusted hazard ratio 0.22, 95% CI
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7 446 0.04 to 1.07, $p=0.06$) (Fig 4B). Two versus seven out of the total randomised patients
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9 447 progressed to confirmed disability in the PLP10 and placebo groups, respectively. No
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11 448 significant differences were observed for groups A or C compared with the placebo group
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13 449 (Fig 4B). The mean change in the EDSS score as a function of visit number is shown in
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15 450 Figure 5.
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452 **MRI**

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23 453 Over two years, the MRI results supported a PLP10-related positive effect as only 29% from
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25 454 the PLP10 group, in contrast to 67% from the placebo group, developed new or enlarging T2
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27 455 lesions (57% relative risk reduction). After excluding the patients on natalizumab, there was
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29 456 an increased relative risk reduction (64%) for PLP10 compared with the placebo, with 29%
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31 457 of patients on PLP10 and 80% on placebo developing new or enlarging T2 lesions (Table 5).
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459 **Safety**

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37 460 Over the course of the 30 month study, no significant adverse events were reported for any
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39 461 group. The only aetiology for the drop-outs was the palatability and smell of the formula
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41 462 preparations in addition to pregnancy. Nausea was reported by two patients. No abnormal
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43 463 values were observed on any of the biochemical and haematological blood tests. No allergic
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45 464 reactions were reported.
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467 **Discussion**

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3 468 In this proof-of-concept, randomised, double-blind clinical trial assessing the safety and
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5 469 efficacy of three variations of a novel nutritional formula in RRMS, we observed a significant
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7 470 association for a formula containing a balanced mixture of specific omega-3 and omega-6
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9 471 PUFAs, MUFAs, SFAs, vitamin A, vitamin E and γ -tocopherol (PLP10) compared with the
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11 472 placebo for both the ARR and the progression of disability in the per-protocol analysis. Our
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13 473 results included analyses pertaining to a total of 42 months of study-collected data, including
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15 474 the 12-month intervention-free treatment extension period. We also observed a high drop-out
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17 475 rate that was mostly the result of formula palatability, a common phenomenon in trials using
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19 476 oily interventions. Interestingly, a statistically significant reduction in the ARR and disability
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21 477 progression was also observed when comparing the ARR of the PLP10 patients in the 24-
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23 478 month period prior to the study with the ARR of the 24 months on-study; the observed
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25 479 differences became larger when the patients who received natalizumab (the currently most
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27 480 potent disease modifier) were excluded. The ARR decreased within a year on PLP10 and
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29 481 remained stable until the study completion. The statistically significant difference in the ARR
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31 482 between patients on PLP10 and those on placebo continued for the 12 month extended period
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33 483 (persistent effect) without a significant difference on the DMT. These clinical findings are
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35 484 supported by the results from the MRI analysis, in which the proportion of patients free from
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37 485 new or enlarging brain T2 lesions was also higher in the PLP10 group than the placebo
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39 486 group. No severe side effects have been reported.
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488 To the best of our knowledge, this study is the first randomised clinical trial assessing the
489 proposed combination of active ingredients in a standardized proportion and dosing scheme
490 for MS treatment designed according to the systems medicine approach. Nutrition is
491 commonly accepted as one of the possible environmental factors involved in the pathogenesis
492 of MS, but its role as a complementary MS treatment is unclear and largely disregarded.⁵¹ It

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3 493 is well known that the majority of the patients suffering from MS do use dietary supplements
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5 494 for a variable length of time.⁵² Dietary antioxidants and fatty acids may influence the disease
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7 495 process in MS by reducing immune-mediated inflammation, oxidative stress and excitotoxic
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9 496 damage.¹² Published data have revealed that healthy dietary molecules have a pleiotropic role
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11 497 and are able to change cell metabolism and down-regulate inflammation by interacting with
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13 498 enzymes, nuclear receptors and transcriptional factors.⁵¹ Current available treatments are the
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15 499 products of reductionism, partially effective and associated with severe side effects.
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17 500 Interferons and glatiramer acetate, the most widely used first-line MS drugs available today,
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19 501 are associated with the least severe side effects among the MS therapies, but they are reported
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21 502 to reduce the ARR only by about one third and with no significant effect on the progression
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23 503 of disability.⁵³ Natalizumab reduces the ARR by 68% and decreases the possibility of
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25 504 disability progression by 43%, with 57% of patients free of new or enlarging T2 lesions on
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27 505 MRI scans, compared with 15% on placebo.⁵⁴ Fingolimod is associated with a 54% ARR
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29 506 reduction (without a significant benefit on the progression of disability). Both natalizumab
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31 507 and fingolimod are second-line drugs associated with severe side-effects.⁵⁵
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35 509 Mehta in a review paper, in 2009, reported different clinical studies on interventions
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37 510 formulated based on the individual aforementioned molecular ingredients or based on a
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39 511 specific ratio of the aforementioned molecular ingredients for MS treatment; although no one
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41 512 was reported using the antioxidant vitamin γ -tocopherol.⁵⁶ In our study, the choice of
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43 513 ingredient proportion and dosing scheme was based upon evidence derived from *in vivo* and
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45 514 *in vitro* data. In the Western diet, the ratio of omega-3 to omega-6 is approximately 1:20–30;
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47 515 in populations that consume fish-based diets, the ratio is approximately 1:1–2.^{57 58} The
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49 516 intervention daily dose was aiming to be, and believed to be, high enough to restore/amplify
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51 517 body-efficient antioxidant activity and ensure cellular membranes lipid profile normalisation
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3 518 (PUFA content) and simultaneously potentiate the involvement of the ingredients in the anti-
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5 519 inflammatory and recovery mechanisms. Dietary fatty acid molecules need an approximately
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7 520 six month period to exert their beneficial effect, and this essential parameter was under
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9 521 consideration for the first time in our study design (normalisation period).⁴⁶ This
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11 522 chronotherapy parameter might be of major importance and is in line with the systems
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13 523 medicine treatment philosophy. We believe that the persistent effect within the post-study
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15 524 period is in agreement with the reported very long washout phase for omega-3 fatty acids,
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17 525 especially DHA, to return to the pre-treatment values.⁴⁶ Considering that omega-3 PUFA
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19 526 supplementation can promote the replacement of AA within the cellular membranes, we can
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21 527 speculate that an increased inflammatory activity can possibly result during the first six
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23 528 months of supplementation.
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30 530 In addition to EPA, DHA, LA, and GLA, PLP10 contained limited quantities of other
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32 531 structural/active PUFAs, specific MUFAs (mostly oleic acid) and SFAs (palmitic and stearic
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34 532 acids), specifically to provide a direct source for neuronal cell membrane rehabilitation and
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36 533 for (re)myelination and neuroprotection because these compounds are all major components,
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38 534 precursors and building blocks of any new physiological myelin and cellular membranes in
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40 535 general. Assembly of the correct molecules into the myelin membrane may be especially
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42 536 critical during active synthesis. If these critical constituents are not directly or indirectly
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44 537 available, amyelination, dysmyelination or demyelination may ensue.⁵⁹ The maintenance of
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46 538 myelin requires continued turnover of its components throughout life.^{60 61}
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52 540 Different factors and molecular entities appear to be part of the possible aetiology for MS,
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54 541 with specific PUFAs and antioxidants found to be key substances related to all known
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56 542 pathogenic and recovery mechanisms. In our study, we further propose that a holistic systems
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3 543 medicine model approach can be applied by synchronised action. First, there is an obvious
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5 544 convenience in administering one formula containing different specific active ingredients.
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7 545 The currently available evidence supports that nutritional interventions would confer a small
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9 546 to medium treatment effect with an accompanying appropriate safety profile.^{12 52 56}
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11 547 Combining these specific active ingredients together with γ -tocopherol and other specific
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13 548 active molecules into one stable formulation is expected to enhance adherence while still
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15 549 offering an appropriate safety profile. A similar approach could not be adopted for
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17 550 pharmaceutical interventions with common and severe adverse events, such as those
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19 551 indicated today for patients with MS. Given the advantages of the simultaneous use and that
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21 552 all the included ingredients have proven individually a valid biological plausibility and have
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23 553 been tested in various settings and under various dose schemes, we also assessed the
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25 554 hypothesis that a novel mixture of these ingredients would have a postulated efficacy attained
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27 555 synergistically through different mechanisms of action.^{52 56} Interestingly, the observed
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29 556 magnitude of the treatment effect cannot be explained by adding up the postulated efficacy
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31 557 estimates of the individual ingredients. Findings from *in vitro* and *in vivo* studies support this
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33 558 notion of proposed synergy although this hypothesis can only be taken forward when the
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35 559 observed treatment effect is validated in various settings and in a larger number of patients.
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39 561 We acknowledge that our study has two considerable limitations: the small sample size and
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41 562 the high drop-out rate. Regarding the sample size, one should bear in mind that this study is a
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43 563 small, phase-2 clinical trial assessing a novel intervention and thus has comparable size in the
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45 564 appropriate literature. Questions taken forward from this trial can be assessed in a larger
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47 565 randomised trial in which appropriate power calculations would be possible, taking into
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49 566 consideration the findings of the present study. The adherence of the subjects is another
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51 567 limitation of our study, but the total duration of the study that covers a total of 42 months
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3 568 follow-up adds power to the results.⁴⁸ We acknowledge that we had to deliver the
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5 569 intervention in the way most frequently associated with low compliance, i.e., an oral, liquid
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7 570 formula, thus triggering maximum intolerance due to taste. Nevertheless, the observed
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9 571 suboptimal compliance is in accordance with the published literature in which clinical trials
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11 572 assessing liquid fatty acid interventions show a weaker adherence compared with clinical
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13 573 trials of pharmaceutical interventions. Indeed, in our study, we consistently recorded the
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15 574 reasons for withdrawal: most of the participants did not discontinue due to safety issues, but
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17 575 rather due to palatability issues. Controlling non-compliance due to palatability issues is by
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19 576 far easier to address compared with non-compliance related to adverse events and can be
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21 577 resolved when optimisation of the formulation is achieved in future trials. At this stage of the
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23 578 development of the intervention, we would by far exceed the cost-effectiveness threshold if
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25 579 we were to invest in improving these features of the intervention. Moreover, we should also
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27 580 note that MS patients are subject to far more frequent and more serious adverse events related
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29 581 to the current standard treatments.
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36 583 As a direct consequence of the low compliance and the loss of power, the performed
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38 584 intention-to-treat analysis was far less robust than intended, and we would then have to take
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40 585 into serious consideration the performed per-protocol analysis. We focused on the per-
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42 586 protocol data analysis because it is the appropriate method to best provide the answer for the
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44 587 proof-of-concept trial-addressed question.²⁴ To validly incorporate the results of the per-
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46 588 protocol analysis into the interpretation of the overall results of the trial, we needed to ensure
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48 589 that the randomisation was not seriously violated due to the exclusion of the non-compliers.
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50 590 The comparison between the baseline characteristics of the patients included in the per-
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52 591 protocol analysis did show a relative balance in the compared groups for known confounders.
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54 592 Nevertheless, the presence of unknown confounders introducing bias to the trial results
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3 593 cannot be excluded despite non- significant differences in the baseline characteristics. As an
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5 594 additional safeguard towards that end, we also performed adjusted analyses for the primary
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7 595 and secondary analyses for important clinical and demographic parameters, i.e., relapses,
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9 596 EDSS, age and DMT.

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14 598 The present preliminary, small-size, randomised, controlled phase II clinical trial provides
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16 599 evidence for a novel nutraceutical formula based on dietary, metabolic, immunological, and
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18 600 neurobiological pathways possibly involved with disease progression in MS. This novel
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20 601 intervention showed signs of efficacy in the observed annualised relapse rate and disability
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22 602 progression. We took the appropriate methodological measures to control for potential
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24 603 sources of bias and to enable a valid interpretation to be reached. We acknowledge that the
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26 604 presence of bias can only be minimised, not excluded, in any clinical research setting and
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28 605 also that random error is always a possible scenario in small trials. Thus, we present the
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30 606 observed results as an additional piece of randomized evidence and anticipate the replication
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32 607 of our study findings in a larger randomised controlled clinical trial.

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1.
Treatment Arms

A†	B (PLP10)†	C†	Placebo†
Intervention: EPA (1,650 mg) / DHA (4,650 mg) / GLA (2,000 mg) / LA (3,850 mg) / total other omega-3 (600 mg)* / total MUFA** (1,714 mg) + total SFA (18:0 160 mg, 16:0 650 mg) / vitamin A (0.6 mg) / vitamin E (22 mg) plus citrus aroma	Intervention: EPA (1,650 mg) / DHA (4,650 mg) / GLA (2,000 mg) / LA (3,850 mg) / total other omega-3 (600 mg)* / total MUFA** (1,714 mg) + total SFA (18:0 160 mg, 16:0 650 mg) / vitamin A (0.6 mg) / vitamin E (22 mg) + pure γ -tocopherol (760 mg) plus citrus aroma	Intervention: pure γ -tocopherol (760 mg) dispersed in pure virgin olive oil (16,137 mg) as delivery vehicle plus citrus aroma	Intervention: Olive oil (pure virgin) plus citrus aroma

* Other omega-3: C18:3n-3 37 mg, C18:4n-3 73 mg, C20:4n-3 98 mg, C22:5n-3 392 mg
 ** MUFA: 18:1 1300 mg, 20:1 250 mg, 22:1 82 mg, 24:1 82 mg
 † Total daily dose 19.5 ml

EPAX1050, EPAX AS, Aalesund, Norway, was used as the source for the omega-3 PUFAs, as re-esterified glycerides from fish body oils; borage seed oil (organic, cold pressed) "Borago officinalis" Goerlich Pharma International GmbH, Edling, Germany, was used as the source for the omega-6 PUFAs, MUFAs and SFAs, as triglycerides. The pure γ -tocopherol was purchased from Tama Biochemical Co. Ltd., Shinjuku-ku Tokyo, Japan; vitamin A, as beta-carotene, from HealthAid Ltd., Middlesex, United Kingdom and the citrus aroma from Givaudan Schwaiz AG, Dubendorf, Switzerland.

Table 1. Intervention ingredients per treatment arm.

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2A.					
Characteristics	Group A (n=20)	Group B† (n=20)	Group C (n=20)	Placebo (n=20)	P- value
Sex					
Female - no. (%)	15 (75)	15 (75)	15 (75)	15 (75)	1.000
Age (yr)					
Mean ± Standard Deviation (SD)	38.0±11.9	36.9±8.4	37.7±8.7	38.1±10.9	0.982
Median (Range)	38.0 (22–65)	37.0 (25–61)	36.5 (24–54)	36.0 (21–58)	
Treatment history					
Patients on DMT- no. (%)	11 (55)	9 (45)	12 (60)	10 (50)	0.875
Pre-treatment disease duration (yr)					
Mean ± SD	9.0±7.6	8.6±4.8	8.6±5.3	7.7±5.7	0.909
Median (Range)	7.5 (2–37)	8.0 (2–20)	8.0 (3–24)	6.5 (2–25)	
Pre-treatment relapses‡					
Mean ± SD	2.33±1.68	2.41±1.73	2.31±1.66	2.10±1.32	0.946
Median (Range)	2.0 (1–6)	2.0 (1–7)	2.0 (1–6)	2.0 (1–4)	
ARR	1.17	1.21	1.16	1.05	0.946
Patients -% with ≤1 relapse	40	45	40	35	
Baseline EDSS score‡					
Mean ± SD	2.52±1.23	2.15±1.05	2.42±1.21	2.39± 0.93	0.775
Median (Range)	2.5 (1.0–5.5)	2.0 (1.0–4.0)	2.5 (0.0–5.0)	2.5 (1.0–4.0)	
2B.					
Characteristics	Group A (n=10)	Group B† (n=10)	Group C (n=9)	Placebo (n=12)	P- value
Sex					
Female - no. (%)	5 (50)	7 (70)	6 (66.6)	10 (83.3)	0.419
Age (yr)					
Mean ± SD	36.6±13.5	34.80±5.4	40.9±8.1	39.8±13.2	0.572
Median (Range)	34.5 (22–65)	34.5 (26–43)	40.0 (29–54)	37.5 (21–58)	
Treatment history					
Patients on DMT- no. (%)	6 (60)	4 (40)	6 (67)	6 (50)	0.949
Pre-treatment disease duration (yr)					
Mean ± SD	9.7±10.0	8.3±5.3	11.3±6.1	8.7± 7.1	0.807
Median (Range)	7.5 (2–37)	8.0 (2–20)	8.0 (4–24)	5.5 (2–25)	
Pre-treatment relapses					

Mean ± SD	2.20±1.47	2.70±1.25	1.78±0.66	1.67±1.37	0.241
Median (Range)	2.0 (1 – 6)	2.5 (1 – 4)	2.0 (1 – 3)	1.5 (1 – 4)	
ARR	1.10	1.35	0.89	0.83	
Patients -% with ≤1 relapse	30	20	33	50	
Baseline EDSS score					
Mean ± SD	2.65±1.37	2.40±1.12	2.11±1.02	2.16±0.96	0.698
Median (Range)	3.0 (1.0–5.5)	2.5 (1.0–4.0)	2.0 (1.0–4.0)	2.0 (1.0–3.5)	

† PLP10 group

‡ Available data at entry baseline (n=18 for group A, n=17 for group B, n=19 for group C, n=19 for group D)

Table 2. Section 2A reports the demographics and baseline disease characteristics for the total randomised population by treatment arm.

Section 2B reports the demographics and baseline disease characteristics of the all-time on-study population by treatment arm. There were no significant between study-group differences at baseline for any characteristic.

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3A.								
Characteristics	Group A (N=10)		Group B† (N=10)		Group C (N=9)		Placebo (N=12)	
End Point	X	Y	X	Y	X	Y	X	Y
Total No. of relapses	22	17	27	8	16	13	20	25
Annual Relapse Rate (ARR)	1.10	0.85	1.35	0.40	0.88	0.72	0.83	1.04
% Reduction Compared with Placebo (primary endpoint) (Ys)¶		-18		-62		-30		N/A
P Value against placebo		0.468		0.024		0.578		
ARR change -% (Y to X)¶		-23		-70		-18		+25
P value against baseline		0.425		0.003		0.578		0.500
X: Total number of relapses for the 24 months pre-treatment (baseline) Y: Total number of relapses for the 24 months on-treatment ¶ Unadjusted estimate								
3B.								
Excluding patients on natalizumab	Group A (N=9)		Group B† (N=10)		Group C (N=9)		Placebo (N=10)	
End Point	X	Y	X	Y	X	Y	X	Y
Total No. of relapses	16	15	27	8	16	13	13	19
Annual Relapse Rate (ARR)	0.88	0.83	1.35	0.40	0.88	0.72	0.65	0.95
% Reduction Compared with Placebo (primary endpoint) (Ys)¶		-13		-58		-24		N/A
P Value against placebo		0.493		0.016		0.412		
ARR change -% (Y to X)¶		-6		-70		-18		+46
P value against baseline		0.857		0.003		0.578		0.354
X: Total number of relapses for the 24 months pre-treatment (baseline) Y: Total number of relapses for the 24 months on-treatment † PLP10 group ¶ Unadjusted estimate								

Table 3. Section 3A reports the two-year primary end points of the ARR for the all-time on-study population by treatment arm and per-cent difference from the placebo. During the 24 mo period on-

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3 treatment, the ARR of group A was 0.85, with an 18% decrease compared with placebo (p=0.468); that
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5 of the PLP10 group was 0.40, with a 62% decrease (p=0.024); and that of group C was 0.72, with a 30%
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7 decrease (p=0.578). This section also reports the comparison of the 24 mo pre-treatment ARR (baseline
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9 ARR) with the 24 mo on-treatment ARR of the all-time on-study population, including patients on
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11 natalizumab.
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16 Section 3B reports the comparison of the 24 mo pre-treatment ARR with the 24 mo on-treatment ARR of
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18 the all-time on-study population excluding patients on natalizumab and the comparison of the ARR
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20 during the 24 mo period on-treatment (primary end point) for each treatment group compared with the
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22 placebo.
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4A.									
Characteristics	Group A (N=8)		Group B† (N=7)		Group C (N=10)		Placebo (N=7)		
	X	Y	X	Y	X	Y	X	Y	
No. of Relapses	20	14	14	14	27	26	20	13	
Annual Relapse Rate (ARR)	1.25	0.88	1.00	1.00	1.35	1.30	1.42	0.92	
X: Total number of relapses for the 24 months pre-treatment Y: Total number of relapses for the 24 months on-treatment									
4B.									
Characteristics	Group A (N=20)		Group B† (N=20)		Group C (N=20)		Placebo (N=20)		
End Point	X	Y	X	Y	X	Y	X	Y	
No. of Relapses	45	34	49	30	46	41	43	41	
Annual Relapse Rate (ARR)	1.13	0.85	1.23	0.75	1.15	1.03	1.08	1.03	
ARR Reduction -% (Y to X)¶	-25		-39		-10		-5		
P value against baseline	0.120		0.005		0.475		0.652		
% Reduction of the ARR Compared with Placebo (Ys)¶	-18		-27		0.0		N/A		
P Value against placebo	0.447		0.121		0.996				
X: Total number of relapses for the 24 months pre-treatment (baseline) Y: Total number of relapses for the 24 months on-treatment ¶ Unadjusted estimate † PLP10 group									

Table 4. Section 4A reports the two year primary end point of relapses based on the study design as reported by the drop-out patients by treatment arm. The most drop-out patients who transferred to disease-modified therapy (DMT) were from group A and the placebo group, with three and two patients, respectively, on natalizumab. These parameters justify the decreased number of relapses recorded within the group A and placebo drop-outs and could affect the ITT analysis in favour of the placebo when the total two-year recorded data are used. For the PLP10 group, 14 relapses were reported at baseline, which remained the same during the two-year study period. For the placebo group, 20 relapses were reported at

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3 baseline and decreased to 13 during the two-year study period. These results are expected
4 because for the PLP10 group, 43% of the drop-outs were under DMT at entry baseline and
5 remained the same until the end of the study, with no patient on natalizumab, but the 57% of
6 the placebo group drop-outs who were under DMT at entry baseline increased to 86% at the
7 end of the study, including two patients on natalizumab.
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16 Section 4B reports the comparison of the 24 month pre-treatment ARR (baseline) with the 24
17 month on-treatment ARR for the total randomised population by treatment arm. The ARR of
18 the PLP10 group was 1.23 at baseline and 0.75 at the end of the study (39% reduction,
19 $p=0.005$), and that for the placebo group was 1.08 at baseline and 1.03 at the end of the study
20 (5% reduction, $p=0.652$). No significant difference was calculated for the other two treatment
21 arms. During the 24 months on-treatment, the PLP10 group presented a 27% reduction in the
22 ARR versus the placebo group ($p=0.121$), with all groups lacking statistically significant
23 results.
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5.					
Characteristics*	Group A (n=10)	Group B PLP10 (n=10)	Group C (n=9)	Placebo (n=12)	P-value Group B vs. Placebo
Annual relapse rate over 1 year**	0.80	0.40	0.78	0.83	
Total number of relapses**	8	4	7	10	
Primary end points					
Annual relapse rate over 2 years (95% CI)**	0.85	0.40 (0.15-0.87)	0.72	1.04	0.024
Total number of relapses**	17	8	13	25	
Excluding patients on natalizumab					
Annual relapse rate over 2 years (95% CI)	0.83	0.40 (0.10-0.79)	0.72	0.95	0.016
Total number of relapses	15	8	13	19	
Secondary end points					
Cumulative probability of sustained progression increase by 1 point on EDSS, confirmed after 6 mo, over 2 years -% **	43	10 (1/10)	24	58 (7/12)	0.019
Excluding patients on natalizumab					
Cumulative probability of sustained progression increase by 1 point on EDSS, confirmed after 6 mo, over 2 years -%	33	10 (1/10)	24	70 (7/10)	0.006
Exploratory Results					
Patient proportion with ≤1 relapse over 2 years -% **	50 (5/10)	90 (9/10)	56 (5/9)	42 (5/12)	0.030
MRI					
Patient proportion with new or enlarging T2 lesions-% **	----	29 (2/7)	----	67 (4/6)	
Excluding patients on natalizumab					
Patient proportion with no new or enlarging T2 lesions-%	----	29 (2/7)	----	80 (4/5)	
DMT (interferons, glatiramer acetate) and natalizumab					
Patient proportion on DMT and natalizumab at the end of 2 years-% **	80 (8/10)†	60 (6/10)	67 (6/9)	75 (9/12) ‡	0.747
* CI denotes confidence interval.					
** Including patients on natalizumab					
† 1 out of 10 on natalizumab					
‡ 2 out of 12 on natalizumab					
Table 5. Clinical end points according to study group for the all-time on-study population.					

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3 668 Acknowledgments: We thank all participating patients. We thank Thyrsos Posporis MD and
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5 669 the central reading centre (Ayios Therissos Medical Diagnostic Centre, Nicosia, Cyprus) as
6
7 670 well as Eleni Eracleous, MD (neuroradiologist) for contributing the MRI scans and their
8
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Article Summary

Article focus:

- The increasing prevalence of multiple sclerosis (MS) combined with the limited efficacy and side effects of the existing treatments urge the development of new, innovative, more effective, safe, and preventive treatment strategies.
- We propose three novel nutraceutical treatment interventions, formulated based on a systems medicine rational through nutritional systems biology; with PLP10 representing the complete composition of the formulation and the other two treatments represent partial formulations.
- We have studied the proposed interventions on 80 relapsing remitting MS patients (four groups of 20 each) for a total of 42 months (including the 12-month extended period) in a randomised, double-blind, placebo-controlled, phase II proof-of-concept clinical trial.

Key messages:

- In this small proof-of-concept, randomised, double-blind clinical trial, the PLP10 treatment statistically significantly reduced the ARR and the risk of sustained disability progression without any reported serious adverse events.
- Overall, a total of 41 (51%) patients completed the 30-month trial. For the per-protocol analysis of primary end point, we observed a 64% relative rate reduction for the PLP10 group (adjusted RRR 0.36, 95% CI 0.15 to 0.87, p=0.024). The cumulative probability of disability progression at two years was 10% in the PLP10 group and 58% in the placebo group (unadjusted log-rank p=0.019).

Strengths and limitations of this study:

- The randomisation, blinding, the use of placebo, the definite inclusion/exclusion criteria and primary/secondary end points, along with the 30 month duration of the study, as well as the inclusion of a 6-month normalisation (chronotherapy) period allow for an appropriate overview of the safety and efficacy of the assessed interventions.
- The small sample size and the high rate of drop-outs (due to the palatability of the formula) are the limitations associated with the present study.

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3 895 **Figure legends**
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6 896 **Figure 1.** Study Flowchart
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9 897 **Figure 2.** Omega-6 and omega-3 PUFAs, their respective metabolic derivatives and their
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11 898 possible effects on inflammation.
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15 899 After consumption, the PUFAs are metabolised via several pathways (not shown) to active
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17 900 compounds that mediate inflammation and to products that promote the resolution of
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19 901 inflammation.
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23 902 Abbreviations: PL, phospholipid; IFN- γ , interferon γ ; IL-2, interleukin 2; NF κ B, nuclear
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25 903 factor kappa B; PGE₂, prostaglandin E₂; PPAR γ , peroxisome proliferator-activated receptor
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27 904 γ ; PUFAs, polyunsaturated fatty acids; TGF β , transforming growth factor β ; TNF, tumour
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29 905 necrosis factor; COX, cyclooxygenase; hydroxyeicosatetraenoic acid; HPETE,
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31 906 hydroperoxyeicosatetraenoic acid; LOX, lipoxygenase; LT, leukotriene; PG, prostaglandin;
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33 907 TX, thromboxane; RXR- γ , retinoid X receptor-gamma; Nrf2, nuclear respiratory factor;
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35 908 MMP, metalloproteinase.
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39 909 **Figure 3.** Panel A demonstrates the ARR of the all-time on-study patients during the 24 mo
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41 910 pre-treatment (baseline ARR) and at different on-study intervals (6, 12, 18, 24 mo) per
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43 911 treatment arm. **
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46 912 Panel B demonstrates the ARR of the all-time on-study population between the 0-6, 6-12, 6-
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48 913 18, and 6-24 mo period intervals for the PLP10 vs. placebo groups. **
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51 914 Panel C demonstrates the ARR of the all-time on-study population for the PLP10 vs. placebo
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53 915 groups at baseline, during the 1st year, and during the 2nd year on-treatment. **
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3 916 Panel D demonstrates the dispersion of relapses throughout the 2-year period of all-time on-
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5 917 study (excluding patients on natalizumab) for PLP10 (n=10) vs. placebo (n=10). The placebo
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7 918 group showed an irregular dispersion of relapses compared with the PLP10 group, with a
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9 919 linear increasing trend, whereas the PLP10 group showed a stabilised linear trend. Using the
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11 920 per-protocol model in which the patients on natalizumab were excluded, the number of
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13 921 relapses could be compared on the same number of patients.
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17 922 ** Including the patients on natalizumab.
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20 923 **Figure 4.** Panel 4A demonstrates the Kaplan–Meier plot of the time to sustained progression
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22 924 of disability among the all-time on-study patients, excluding the patients on natalizumab,
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24 925 receiving interventions A, PLP10 and C compared with placebo. PLP10 reduced the risk of
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26 926 the sustained progression of disability by 86% over two years (p=0.006). Intervention
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28 927 formula A reduced the risk of the sustained progression of disability by 53% (p=0.266), and
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30 928 intervention formula C, by 67% (p=0.061).
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34 929 Panel 4B demonstrates the Kaplan–Meier plot of the time to sustained progression of
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36 930 disability among the ITT population receiving interventions A, PLP10 and C compared with
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38 931 placebo. PLP10 reduced the risk of the sustained progression of disability by 71% over two
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40 932 years (p=0.052, trend). Intervention formula A reduced the risk of the sustained progression
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42 933 of disability by 22% (p=0.727), and intervention formula C, by 40% (p=0.447).
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46 934 **Figure 5.** Mean change in the expanded disability status scale score as a function of visit
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48 935 number. The values are expressed as the mean ± standard error of the mean (s.e.m.)
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51 936 ¶ Including patients on natalizumab
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54 937 ¶¶ Excluding patients on natalizumab
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7 **A novel oral nutraceutical formula of omega-3 and omega-**
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10 **6 fatty acids with vitamins (PLP10) in relapsing remitting**
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13 **multiple sclerosis: a ~~randomized~~randomised, double-blind,**
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16 **placebo-controlled proof-of-concept clinical trial**

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19 **Marios C. Pantzaris***, **George N. Loukaides**, **Evangelia E. Ntzani**, **Ioannis**

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21 **S. Patrikios***

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24 * Both M.C.P and I.S.P are the first authors and both are the corresponding authors
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26
27

28 The Cyprus Institute of Neurology and Genetics (CING) 1683 Nicosia, Cyprus Marios C.

29 Pantzaris Senior Consultant Neurologist Neurology Clinic C and PALUPA Medical Ltd., The

30 Cyprus Institute of Neurology and Genetics (CING) 1683 Nicosia, Cyprus George N.

31 Loukaides Clinical Dietitian/Nutritionist Neurology Clinic and PALUPA Medical Ltd.,

32 University of Ioannina School of Medicine (UISM) 45110 Ioannina, Greece Evangelia E.

33 Ntzani Assistant Professor Clinical and Molecular Epidemiology Unit, Department of

34 Hygiene and Epidemiology, PALUPA Medical Ltd at The Cyprus Institute of Neurology and

35 Genetics (CING) 1683 Nicosia, Cyprus Ioannis S. Patrikios Senior Research Scientist,

36 visiting Professor at European University Cyprus Nicosia, Cyprus, Department of Health and

37 Science. Correspondence should be addresses to e-mail: I.Patrikios@euc.ac.cy or

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71
72 **Results** A total of 41 (51%) patients completed the 30-month trial. Overall, for the per-
73 protocol analysis of the two-year primary end point, 8 relapses were recorded in the PLP10
74 group (n=10) (0.40 ARR) versus 25 relapses in the placebo group (n=12) (1.04 ARR),
75 representing a 64% adjusted relative rate reduction for the PLP10 group (RRR 0.36, 95% CI
76 0.15 to 0.87, p=0.024). In a subgroup analysis that excluded patients on monoclonal antibody
77 (natalizumab) treatment, the observed adjusted RRR became stronger (72%) over the two
78 years (RRR 0.28, 95% CI 0.10 to 0.79, p=0.016). The per-protocol analysis for the
79 secondary outcome at two years, the time to disability progression, was significantly longer
80 only with-for PLP10. The cumulative probability of disability progression at two years was
81 10% in the PLP10 group and 58% in the placebo group (unadjusted log-rank p=0.019). In a
82 subgroup analysis that excluded patients on natalizumab the cumulative probability of
83 progression was 10% for the 10 patients in the PLP10 group and 70% for the 12 patients in
84 the placebo group, representing a relative 86% decrease in the risk of the sustained
85 progression of disability in the PLP10 group (unadjusted log-rank p=0.006; adjusted hazard
86 ratio, 0.11; 95% CI 0.01 to 0.97, p=0.047). No adverse events were reported. Interventions A
87 (10 patients) and C (9 patients) showed no significant efficacy.

88
89 **Conclusions** In this small proof-of-concept ~~randomized-randomised~~ double-blind clinical
90 trial, the PLP10 treatment significantly reduced the ARR, and the risk of sustained disability
91 progression without any reported serious adverse events. Larger studies are needed to further
92 assess the safety and efficacy of PLP10.

93
94 **Trial registration** International Standard ~~Randomized-Randomised~~ Controlled Trial, number
95 ISRCTN87818535.

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122 synergistic effect and provide ~~to give~~ a comprehensive, sustainable treatment effect
 123 (Supplementary Information Methods 1).

124 ~~long, holistic and effective treatment (Supplementary Information Methods 1).~~

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126 The polyunsaturated fatty acids (PUFA) composition of membrane phospholipids plays an
 127 ~~direct important~~ role in immune- and non-immune-related inflammation. PUFA and
 128 antioxidant deficiencies along with a decreased cellular antioxidant defense mechanisms have
 129 been reported in MS patients.¹¹ The cause of ~~these~~ PUFA deficiencies is not entirely clear and
 130 may involve metabolic and nutritional alterations.¹¹

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131 ~~Increased or uncontrolled inflammation contributes to several different acute and chronic~~

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132 ~~diseases, and it is characterized by the production of inflammatory cytokines, arachidonic~~

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134 Increased or uncontrolled inflammation contributes to several different acute and chronic
 135 diseases, and it is characterized by the production of inflammatory cytokines, arachidonic
 136 acid (AA)-derived eicosanoids (prostaglandins [PGs], thromboxanes [TXs], leukotrienes
 137 [LTs], and other oxidized derivatives), and other inflammatory agents such as reactive
 138 oxygen species (ROS), nitric oxide (NO), and adhesion molecules (Fig 2).¹² ~~During~~
 139 ~~inflammation, glutamate homeostasis is altered by the release of increased quantities of~~
 140 ~~glutamate by activated immune cells, which can result in the over-activation of glutamate~~
 141 ~~receptors and, in turn, excitotoxic oligodendroglial death. During inflammation glutamate~~
 142 ~~homeostasis is altered by activated immune cells releasing increased quantities of glutamate~~
 143 ~~that can result in over activation of glutamate receptors and in return excitotoxic~~
 144 ~~oligodendroglial death.~~^{7, 12, 13} ~~As such, a~~mong others, membrane-related pathology,
 145 immune-mediated inflammation, oxidative stress, and excitotoxicity provide potentially
 146 useful combined targets for intervention in MS.

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147
148 *In vitro* and *in vivo* studies have demonstrated that dietary eicosapentaenoic acid (EPA),
149 docosahexaenoic acid (DHA), linoleic acid (LA), and gamma (γ)-linolenic acid (GLA) can be
150 implicated and modulate almost all known complex network of events and pathways
151 repertoire in MS pathophysiology. The Brain membrane fatty acid composition can be
152 modified with dietary supplementation, but the process has been showed-shown to be age
153 dependent (#-takesing much longer in adults versus developing brains) as-well-asand possibly
154 dependent on the quantities-quantity of the dietary/supplemented PUFAs.¹⁴ Both human and
155 animal studies proved that diets high in DHA and EPA can increase the proportion of these
156 PUFAs in the membranes of inflammatory cells and can also reduce the levels of AA, a
157 stress-related biomarker.^{12, 14-15} The anti-inflammatory properties of omega-3 include the
158 production of PGs and TXs of the 3-series and LTs of the 5-series (Fig 2).^{14, 16} Resolvins and
159 protectins are biosynthesized-biosynthesised from omega-3 fatty acids via cyclooxygenase-
160 2/lipoxygenase (COX-2/LOX) pathways and they promote control of inflammation in neural
161 tissues (Fig 2).¹⁷⁻²¹ T-cell proliferation in acute and chronic inflammation can be reduced by
162 supplementation with either omega-6 or omega-3 PUFAs.²² Furthermore, vitamin E is an
163 important antioxidant that can interrupt the propagation of free radical chain reactions.^{23, 24}
164 Vitamin E (alpha-tocopherol, an isoform of vitamin E) efficiently detoxifies hydroxyl,
165 perhydroxyl and superoxide free radicals, whereas.²⁴ However, γ-tocopherol (another
166 isoform of vitamin E) seems-appears to be more efficiently implicated in trapping NO
167 radicals.^{24, 25} In addition alpha-tocopherol exerts non-antioxidant properties, including the
168 modulation of cell signaling and immune functions, regulation of transcription, and induction
169 of apoptosis.²⁶

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7 171 Moreover, omega-3 fatty acids electrophilic derivatives formed by COX-2, in activated
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9 172 macrophages can stimulate the nuclear respiratory factor (Nrf2), ~~which inducing induces~~ the
10
11 173 transcription of neuroprotective and antioxidant-related genes, and can activate the
12
13 174 peroxisome proliferator-activated receptor (PPAR) ~~γ~~ for ~~an~~ anti-inflammatory response.²⁷⁻²⁹
14
15 175 In animal studies, EPA and DHA proved to be endogenous ligands of RXRs, with positive
16
17 176 effects on neurogenesis.³⁰ Additionally, in 2008, Salvati and coworkers reported ~~evidense~~
18
19 177 ~~evidence~~ of accelerated myelination in DHA- and EPA-treated animals.³² Moreover, DHA
20
21 178 and EPA ~~are-have been~~ reported to significantly decrease the levels of metalloproteinases
22
23 179 (MMP) -2, -3, -9, and -13, ~~with-which have~~ a significant role in the migration of lymphocytes
24
25 180 into the ~~central nervous system (CNS)~~ by inducing ~~the~~ disruption of the blood brain barrier
26
27 181 (BBB), an important step in the formation of MS lesions.³³⁻³⁹
28
29 182
30 183 Based on the above ~~observations~~, specific PUFA and antioxidant vitamins fulfill the criterion
31
32 184 of biologic plausibility and have the potential to diminish ~~the severity and activity of~~ MS
33
34 185 symptoms ~~severity and activity~~, ~~potentially~~ even promoting recovery (remyelination).¹¹
35
36 186 ~~Overall, PLP10 contains multiple ingredients (omega 3, omega 6 and other fatty acids and~~
37
38 187 ~~vitamins) potentially able to modulate key interconnected components (i.e. genes, proteins)~~
39
40 188 ~~and structural molecules (i.e. cellular membrane lipids, receptors) within the functional~~
41
42 189 ~~network of events of MS pathogenesis.~~⁴⁰
43
44 190
45 191 ~~We report here This is a~~ ~~randomized-randomised~~ phase II, single-center, double-blind,
46
47 192 placebo-controlled, proof-of-concept clinical trial evaluating the therapeutic ability of a
48
49 193 nutraceutical formula (with PLP10 representing the complete composition of the formulation)
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51 194 and of two other interventions (A and C) consisting of PLP10-constituent partial fractions
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195 containing ingredients for the aforementioned substance categories on relapsing remitting
196 (RR) MS patients.

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199 **Methods**

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200 **Patients**

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201 The eligibility criteria were an age of 18 to 65; a diagnosis of RRMS according to the

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202 McDonald criteria; a score of 0.0 to 5.5 on the Expanded Disability Status Scale (EDSS), a

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203 rating that ranges from 0 to 10, with higher scores indicating more severe disability; MRI

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204 showing lesions consistent with MS; and at least one documented clinical relapse; and either

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205 receiving or not a disease modifying treatment (DMT) within the 24 months period before

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206 beginning (enrollment) inof the study. Patients were excluded because of a recent (<30 days)

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207 relapse, prior immunosuppressant or monoclonal antibodies-antibody therapy, pregnancy or

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208 nursing, other severe disease compromising organ function, progressive MS, history of recent

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209 drug or alcohol abuse, use of any additional food supplement, vitamins, or any form of

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210 PUFA, and history of severe allergic or anaphylactic reactions or known specific nutritional

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211 hypersensitivity. No monitor or limitations on the patients' daily diearyt habits were included

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212 considered in the study design since because the high quantities of the ingredients within the

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213 formula, s daily dosage ecould not be significantly affected or spoiled by any particular

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214 dietary pattern by any confounding factors within any known global daily food diet (see

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215 procedures, treatment regimen and end points).

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216

217 The study was conducted in accordance with the standards of the International Conference of

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218 Harmonization Guidelines for Good Clinical Practice. The protocol was developed by the

219 investigators and it was approved by the Cyprus National Bioethics Committee and was

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7 220 | overseen by an independent safety-monitoring committee evaluating the safety and overall
8
9 221 | benefit-risk profiles. The adherence of care providers with the protocol was assessed by an
10
11 222 | external committee assigned by the funder of the project through reviews of case report
12
13 223 | forms. All patients gave written informed consent at the time of enrolment.

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16 225 | ~~Randomization~~ ~~Randomisation~~ and masking

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17
18 226 | Patients were randomly assigned to four intervention groups in a 1:1:1:1 ratio stratified by
19
20 227 | gender (women to men, 3:1). ~~Randomization~~ ~~Randomisation~~ was facilitated by a lottery-type
21
22 228 | pool of numbered balls. Patients were randomly assigned to treatment in blocks of four by
23
24 229 | flipping a coin as follows: for the first two drawn balls, heads stratified them to the groups
25
26 230 | A/B and tails stratified them to the groups C/D. The other two balls were stratified
27
28 231 | accordingly. A second toss of the coin assigned the two patients to group A (head)/B (tail) or
29
30 232 | group C (head)/D (tail). The ~~randomization~~ ~~randomisation~~ scheme was generated, performed
31
32 233 | and securely stored by the Helix Incubator Organization of Nicosia University (HIONU).

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33
34 234 |
35 235 | The interventions had identical appearance and smell and were kept in dark bottles (15 daily-
36
37 236 | dose portions/bottle) under nitrogen bed and labeled by HIONU with code numbers,
38
39 237 | unidentifiable-blinded for both patients and investigators. Study data were collected by the
40
41 238 | investigators and saved by the HIONU, which that also held the blinded codes of the study.

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42
43 239 | All study personnel involved in the conduct of the study were blinded throughout the study.

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45 240 | The treating/examining physician, all other investigators, the pharmacist, the
46
47 241 | neuroradiologist and all patients were masked to treatment allocation.

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51 243 | Procedures and end points

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7 244 The specific omega-3 (~~re-esterified glycerides~~) and omega-6 (~~glycerides~~) raw materials were
8
9 245 purchased according to the required interventions' PUFA-fraction specification (molecular
10
11 246 structure, quantity/ratio and quality) with vitamin E (alpha-tocopherol) used as antioxidant
12
13 247 ~~stabilizer-stabiliser~~ by the supplier. The vitamins and masking aroma were purchased
14
15 248 separately. The mixing of fractions to the final required intervention-composition
16
17 249 specification was always performed by the same team of scientists under the supervision of
18
19 250 the involved medical biochemist and lipidology specialist ~~and~~ under appropriate conditions
20
21 251 every six months. ~~The~~ interventions were ~~stored~~-refrigerated in ~~the~~ dark until use. See Table
22
23 252 1 and Supplementary Information Methods 1 and 2 for ~~the a detailed description of the~~
24
25 253 ~~interventions specification detailed description, and study/intervention rational.~~
26
27
28 254
29 255 ~~The~~ participants were randomly assigned to receive ~~the following~~: ~~in~~ group A, a daily dose
30
31 256 of a 19.5ml mixture of EPA (1,650mg) / DHA (4,650mg) / GLA (2,000mg) / LA (3,850mg) /
32
33 257 total other omega-3 (600mg) / total MUFA (1,714mg) + total SFA (18:0 160mg, 16:0
34
35 258 650mg) / vitamin A (0.6mg) / vitamin E (22mg) plus citrus-aroma (intervention A); ~~in~~ group
36
37 259 B PLP10, a daily dose of a 19.5ml mixture of EPA (1,650mg) / DHA (4,650mg) / GLA
38
39 260 (2,000mg) / LA (3,850mg) / total other omega-3 (600mg) / total MUFA (1,714mg) + total
40
41 261 SFA (18:0 160mg, 16:0 650mg) / vitamin A (0.6mg) / vitamin E (22mg) plus pure γ -
42
43 262 tocopherol (760mg) plus citrus-aroma (intervention B); ~~in~~ group C, a daily dose of a 19.5ml
44
45 263 mixture of pure γ -tocopherol (760mg) dispersed in pure virgin olive oil (16,137 mg) plus
46
47 264 citrus-aroma (intervention C); and ~~in~~ group D (placebo), a daily dose of a 19.5ml mixture of
48
49 265 pure virgin olive oil (16,930mg) plus citrus-aroma (intervention D) (Table 1). The
50
51 266 ~~institution's~~ pharmacist ~~of the institution~~ was responsible for the appropriate storage and
52
53 267 handling of the interventions ~~to for~~ the individual participants. The interventions were taken
54
55 268 orally once daily 30 minutes before dinner by ~~using~~ a dosage calibrated cup for 30 months.

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269 The ingredients, ratio and dose ~~have been were~~ selected based on their biophysical
 270 interrelation ~~to with~~ the total known multiple MS ~~causing causative~~ factors, their biochemical
 271 importance and the role ~~they were~~ expected to play in the normalisation and treatment of the
 272 involved complex network of events in the disease pathophysiology. Moreover, the high
 273 ~~intervention intake~~ dosage was ~~selected with the aim of optimising the body composition of~~
 274 ~~omega-3 to omega-6 PUFAs to a 1:1 wt/wt ratio irrespective of dietary habits and~~
 275 ~~geographical origin.~~
 276 ~~used to overcome any abnormal dietary accumulation of related agents as a result of patients'~~
 277 ~~food intake habits, irrespective of geographical origin, in relation to the daily consumption~~
 278 ~~ratio of the total fatty acid intake; in order to end up with omega 3 to omega 6 PUFA~~
 279 ~~indicated physiological body ratio composition of 1:1 wt/wt.~~

281 The period ~~beginning~~ from July 1st 2007 (enrollment) ~~until to~~ December 31st 2007 (~~entry~~
 282 ~~baseline~~) was used ~~for as the normalization-normalisation~~ period. This six-month
 283 ~~normalization-normalisation~~ period would allow the interventions' ~~agents~~ to exert their
 284 beneficial effects ~~as (for the incorporation/normalization of cell membranes by oral PUFA,~~
 285 ~~since they oral PUFAs~~ need four to six months to ~~achieve exert~~ pivotal action on immune and
 286 neural cells, ~~a~~ correction of antioxidant ~~deficiency deficiencies~~ and body PUFA
 287 ~~redistribution~~, and ~~an~~ optimal normalization of ~~the~~ EPA and DHA ~~levels/ratio~~.⁴¹⁻⁴³ The study
 288 was completed on December 31st 2009 (~~30 months~~) and the recording of relapses continued
 289 until December 31st 2010 (~~42 months~~). ~~More-Overall, clearly~~ the study included ~~the a~~
 290 "normalization period" (July 1st 2007 to Dec 31st 2007), ~~the an~~ "on treatment" period (Jan 1st
 291 2008 to Dec 31st 2009) and ~~the a~~ 12-month "post study ~~extended monitoring~~ period" (Jan 1st
 292 2010– Dec 31st 2010).

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294 Depending on their clinical status and in accordance with ~~the common practice~~ ~~the ethical~~
 295 ~~issues governing clinical trials, the~~ participants continued receiving their ~~indicative~~ ~~indicated~~
 296 regular ~~treatment~~, with persistent evaluation for any side-effects and adverse events.
 297 Clinical ~~assessments~~ ~~assessmen visits~~ were scheduled at ~~entry~~ baseline, ~~and~~ 3, 9, 15, 21 and
 298 24 months on-treatment. ~~The P~~patients were also clinically examined by the treating
 299 neurologist within 48 hours after the onset of new or recurrent neurologic symptoms.
 300
 301 The primary end point was the ~~annualized relapse rate (ARR)~~ at two years. A relapse was
 302 defined as new or recurrent neurologic symptoms not associated with fever or infection that
 303 lasted for at least 24 hours and ~~was~~ accompanied by new neurologic signs. Relapses were
 304 treated with methyl-prednisolone at a dose of 1g intravenous per day, for three days followed
 305 by prednisone orally at a dose of 1mg/kg of weight per day on a tapering scheme for three
 306 weeks. The secondary end point at two years was the time to ~~confirmed~~ disability
 307 progression, defined as an increase of 1.0 or more on ~~the~~ EDSS ~~and~~ confirmed after six
 308 months. ~~(p~~Progression could not be confirmed during a relapse) ~~and t~~The final EDSS score
 309 was confirmed six months after the end of the study. ~~A post-hoc analysis was performed to~~
 310 assess ~~ing~~ the proportion of patients free from new or enlarging T2 lesions on brain MRI
 311 scans at the end of the study for the per-protocol participants of the group receiving the
 312 ~~highest most~~ effective intervention versus placebo. ~~This C~~comparison was made versus the
 313 available archival MRI scans up to three months before the enrolment date. ~~The~~ MRI scans
 314 were performed and blindly analyzed at an MRI evaluation centre. The patients ~~continued to~~
 315 ~~be followed~~ ~~were monitored~~ for ~~an~~ additional 12 months after completion of the trial and
 316 relapses were recorded. ~~Finally, The~~ patients were strongly encouraged to remain in the study
 317 for follow-up assessments even if they had discontinued the study drug.
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319 Blood samples were collected from all ~~randomized-randomised~~ patients at the time of
 320 enrolment, at every scheduled clinical assessment and during relapses. To ~~check-evaluate the~~
 321 ~~individual~~ compliance ~~with intake~~, the fatty acids composition of ~~the~~ patients' red blood
 322 cells' membranes was determined, by gas chromatography, according to a standard protocol.
 323 The fatty acid analyses were performed after study termination and thus did not influence the
 324 blinding. Safety measures were assessed from the time of enrollment until 12 months
 325 following the study completion. Haematological and biochemical tests were performed at
 326 enrolment and every 12 months, including full blood count, renal and liver function tests, and
 327 proteins, cholesterol, triglycerides, glucose and electrolyte levels.

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329 The involved neurologist was experienced with more than 20 years in practice. ~~He was and~~
 330 trained to standardise ~~the~~ EDSS scoring procedures, examined patients, made all medical
 331 decisions, determined the EDSS score and reviewed ~~the~~ adverse effects or side-effects. The
 332 medical biochemist, ~~who was a~~ specialist ~~on-in~~ lipidology and immunology, and the
 333 registered clinical dietitian, ~~were both~~ members of the ~~investigator-investigative~~ team ~~were~~
 334 ~~experienced~~ with more than 25 years in practice. ~~The P~~ patients were able to contact the
 335 ~~involved~~ neurologist at any time if there was any adverse event, side-effect or allergic
 336 reaction. The study drug was not ~~suspected-expected~~ to have any clinical or laboratory
 337 adverse effects different from ~~those of the~~ placebo that could disturb the double-blind nature
 338 of the trial. Therefore, the ~~same~~ study-neurologist functioned as both the treating and
 339 evaluating physician.

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341 The whole procedure followed the clinical trial guidelines as required by the USA Food and
 342 Drug Administration, European Medicines Agency, and the Committee for Medicinal
 343 Products for Human Use.⁴⁴

344

345 Statistical analysis

346 Power calculations could not be ~~done-performed~~ before the study because of the lack of
347 information from previous studies on the potential effect sizes. In 2005, the prevalence of MS
348 in Cyprus (600,000 population) was 120/100.000. Based on the aforementioned MS patients'
349 numbers of our country and the ~~centre-of-reference~~ centre, the CING, we were able to enrol
350 ~~the~~ 20% of the total RRMS patients eligible for treatment ~~in the trial~~. The sample size was
351 strictly based on ~~this-the~~ subjects' availability ~~parameter~~ and the novelty of the assessed
352 intervention.

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354 The ~~B~~baseline characteristics were compared across all intervention groups by ANOVA or
355 the Kruskal-Wallis rank test for continuous variables and by mean Fisher's exact test for
356 categorical variables, as appropriate.

357

358 For the primary outcome, the ARR was analysed in a pair-wise fashion for the active
359 interventions compared with the ~~to~~ placebo using negative binomial regression models
360 adjusted for the number of relapses within two years ~~before baseline~~, the EDSS score at
361 baseline and DMT. The relapse rate was calculated as the total number of relapses divided by
362 the total number of patient-years followed for each treatment group. ARR differences were
363 also calculated among all comparable parameters and reported as the per-cent difference.

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365 For the secondary end-point ~~outcome~~, the time to disability progression, Kaplan–Meier
366 curves were constructed. The ~~P~~progression ~~to-of~~ disability and time thereof was compared in
367 a pair-wise fashion for the active interventions versus placebo by the log-rank test in the main
368 analysis and by the Cox proportional-hazards models with adjustment for the baseline EDSS

369 score, age and DMT in the supportive analysis. ~~Each test was performed with a significance~~
 370 ~~level of 0.05.~~ Multivariate models considered all variables with P <0.1 ~~on in the~~ univariate
 371 models. There was no overt violation of the proportionality assumption.

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373 Both, per-protocol and intention to treat (ITT) analyses were performed ~~for~~ for different sets of
 374 research questions to be answered, and both are reported. Missing data of the five ~~lost to~~
 375 ~~follow~~ patients ~~lost to follow-up~~ were imputed by ~~use of~~ the last-observation-carried-forward
 376 (LOCF) approach. Due to the proof-of-concept design of the study, the considerable non-
 377 adherence rate (49%) and the ~~resulting~~ interpretation issues ~~caused thereof~~ regarding the ITT
 378 analysis, the per-protocol analysis ~~was considered to be the~~ ~~ing~~ more informative and
 379 appropriate method approach to answer the research ~~addressed~~ questions ~~addressing of the~~
 380 efficacy of the interventions when subjects ~~were~~ continuously ~~following~~ followed the
 381 protocol. All statistical analyses were well defined a priori. All analyses were performed with
 382 STATA SE 10.0 (College Station, TX, USA). P-values are two-tailed.

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384 **Role of the funding source**

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385 The funders had no role in study design, data collection and analysis, decision to publish, or
 386 preparation of the manuscript. All members of the writing group had full access to all study
 387 data and contributed to its interpretation and prepared, reviewed, and approved the
 388 manuscript for submission. All authors had final responsibility for the decision to submit the
 389 paper for publication.

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391 **Results**

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392 **Study population**

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393 ~~From July 2007 through December 2010 (including the 12 month extended period), a total~~
 394 ~~of 80 MS patients were randomly assigned to a study group at the CING (tertiary~~
 395 ~~neurological center).~~

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397 Among the 80 patients, 20 patients were randomly assigned to each of the three groups to
 398 receive the interventions, and 20 to receive placebo (Fig 1). ~~The B~~ baseline characteristics of
 399 both the ITT and the per-protocol populations were similar across groups (Table 2A and 2B).

400 All patients ~~that who~~ dropped out ~~had a~~ completed ~~the~~ follow-up until ~~the~~ study completion
 401 and were included in the ITT analyses (Table 4). Five patients were lost to follow-up before
 402 their first scheduled visit. ~~Tand~~ two other patients ~~who that~~ dropped-out before their first
 403 scheduled visit progressed to secondary progressive MS. Fifteen patients dropped-out
 404 without successfully completing the “normalization” period, including five pregnancies.

405 Another 17 patients dropped-out early after ~~the~~ entry baseline. Seven patients ~~that who~~
 406 dropped out were given monoclonal antibody treatment (natalizumab). Overall, a total of 41
 407 (51%) patients completed the 42-month study (~~July 2007 through December 31st 2010,~~

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408 ~~including the 12 month extended period), where~~ one patient from group A and two from the
 409 placebo group transferred on natalizumab, and 39 (49%) patients either withdrew (dropp-ed
 410 out) or lost to follow. ~~The R~~ reasons for ~~study interventions~~ discontinuation are listed in
 411 Figure 2.

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412 **Efficacy**

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413 **Relapses**

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415 As a proof-of-concept trial, we primarily needed to answer whether the interventions were
 416 effective for those MS patients who adhere to the assigned treatment, ~~which was~~ the per-
 417 protocol analysis.⁴⁵ For ~~the sake of~~ methodological comprehensiveness, we also ~~present~~

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7 418 | performed the ITT analysis as a secondary analysis, to answer ~~a~~ different questions; ~~that were~~
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9 419 | complementary to our core hypothesis, such as; ~~like~~ what happened to all MS patients who
10
11 420 | were placed on the interventions (the effect of assignment).⁴⁵
12
13 421 |

14 422 | ~~Regarding~~ In the per-protocol analysis, during the first year of treatment, the ARR was 0.80,
15
16 423 | 0.40, 0.78 and 0.83 for the four intervention groups respectively. During the second year, the
17
18 424 | ARR was 0.90, 0.40, 0.67 and 1.25 for the four intervention groups respectively. Overall, for

19
20 425 | ~~the two-year primary end point~~, 8 relapses were recorded for the 10 patients in PLP10 group
21
22 426 | (0.40 ARR) versus 25 relapses for the 12 patients ~~in on the~~ placebo (1.04 ARR), a 64%
23
24 427 | adjusted relative rate reduction (RRR) for the PLP10 group (RRR 0.36, 95% confidence

25
26 428 | interval [~~CI~~] 0.15 to 0.87, p=0.024) (Tables 3A, 5 and Fig 3A and 3C). ~~After~~ ~~Excluding~~
27
28 429 | patients on monoclonal antibody (natalizumab) treatment, the observed adjusted RRR

29
30 430 | became stronger (72%) over the two years (RRR 0.28, 95% CI 0.10 to 0.79, p=0.016, Tables
31
32 431 | 3B and 5). Pair-wise comparisons for the other two groups against placebo did not yield

33
34 432 | statistically significant results (Tables 3A, 3B). The proportion of patients with ≤ 1 relapse for
35
36 433 | the two years on-study was higher in the PLP10 group than in the placebo group (90% versus
37
38 434 | 42%, p=0.030, Table 5). Seeking to ~~investigate~~ further investigate the observed difference,

39
40 435 | we compared the relapse rate during the 24 months before the entry into the study to the 24
41
42 436 | months on-treatment for each intervention group. We observed a statistically significant

43
44 437 | ~~relative reduction in the ARR (70%) only in the PLP10 group (RRR 0.30; 95% CI 0.14 to~~
45
46 438 | 0.65, p=0.003, Table 3A); within-group comparisons for the ~~three other groups~~ ARR

47
48 439 | reduction of the three other groups was were not significant and remained not significant

49
50 440 | when the natalizumab--treated patients were further excluded from the analysis. The effect of
51
52 441 | PLP10 through time at different time-windows versus placebo for all-time on-study patients

53 442 | is shown in Figures 3A to 3D. ~~Although the~~ ~~The~~ ARR analysis; within time-windows; was not
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7 443 an assigned endpoint, ~~but~~ it could help ~~in-with~~ the process of evaluating parallel information,
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9 444 ~~such as the time needed for a specific treatment intervention activity to be evident, as well as~~
10
11 445 the efficacy profile through time. PLP10 reached ~~its~~ maximum effect within ~~a-one~~ year on-
12
13 446 treatment (counting from the entry baseline) and remained stable ~~afterwards~~ at an ARR of
14
15 447 0.4, ~~displaying a steadily reduced ARR with long- with some~~ free-relapse time-windows.
16
17 448 ~~These group B characteristics are considered important parameters of a successful MS~~
18
19 449 ~~treatment where the rule than the exception is the heterogeneity among patients' disease~~
20
21 450 ~~evolution. Specifically,~~ Figure 3D demonstrates the dispersion of relapses throughout the 2-
22
23 451 year period of all-time on-study (excluding patients on natalizumab) of PLP10 (n=10) versus
24
25 452 placebo (n=10). ~~The p~~Placebo group, in line with the existing knowledge of how relapse
26
27 453 history works in relation to future relapses ~~on-in~~ MS patients (contagion phenomenon),
28
29 454 ~~showed indicates~~ the expected ~~linearly increased~~ trend of ~~the increased~~ relapse incidences.⁴⁶
30
31 455 The same phenomenon was true for ~~the~~ groups A and C. Finally, during the 12 month post-
32
33 456 study extended period, ~~the (January 1st 2010 to December 31st 2010) all time~~ on-study
34
35 457 patients ~~that-who~~ received PLP10, showed ~~a~~ persistent benefit in the ARR compared ~~with~~
36
37 458 ~~the~~ placebo (six relapses for the 10 subjects within PLP10 group, 0.6 ARR versus 19 for the
38
39 459 ~~12~~ subjects within placebo group, 1.58 ARR) indicating a statistically significant 62%
40
41 460 adjusted relative rate reduction in the ARR for the PLP10 group (RRR 0.38, 95% CI 0.12 to
42
43 461 0.99, p=0.046).

44
45 463 Regarding the ITT analysis, ~~within PLP10 group, none of the nine drop-out patients changed~~
46
47 464 ~~to natalizumab and two out of nine discontinued because of pregnancy, whereas two out of~~
48
49 465 ~~seven drop-out patients from the placebo group changed to natalizumab (a total of four~~
50
51 466 ~~patients within the placebo arm population were on natalizumab, including the two patients~~
52
53 467 ~~that transferred while all time on study versus none within PLP10 group (Supplementary~~

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Information Fig 1). As reported, natalizumab reduces ARR by 68%, decreases the possibility of disability progression by 43% with 57% of patients free of new or enlarging T2 lesions on MRI scans compared to 15% on placebo.⁴⁷ The relapses of the drop-out patients are reported in Table 4A. As expected, no statistically significant differences in the ARR were calculated for the comparison of any group versus placebo for the 24 months on-treatment, with a 0.75 ARR within PLP10 (30 relapses) and 1.03 ARR within placebo (41 relapses) group, a 27% ARR reduction (Table 4B). The ITT population on DMT and/or on natalizumab is shown within the Supplementary Information Fig 1. Interestingly, despite the high non-adherence rate, there was a statistically significant difference for the comparison of the ARR in the 24 months before entry baseline to the 24 months on-treatment for the PLP10 group (RRR 0.45, 95% CI 0.26 to 0.78, p=0.005).

Disability progression

Regarding In the per-protocol analysis, at two years, the time to disability progression, with confirmation after six months (secondary end point) was significantly longer only with PLP10. The cumulative probability of disability progression was 10% in the PLP10 group and 58% in the placebo group (p=0.019) (Supplementary Information Fig 2). After excluding excluding the patients on natalizumab, there was again a n-increased statistically significant difference between the PLP10 and the placebo groups for the same analysis (p=0.006) (Fig 4A). At two years, the cumulative probability of disability progression was 10% in the PLP10 group and 70% in the placebo group, which represents a decrease of 60 percentage points or a relative 896% decrease in the risk of the sustained progression of disability within within the PLP10 group (adjusted hazard ratio, 0.11; 95% CI 0.01 to 0.97, p=0.047). One versus seven out of ten patients progressed to confirmed disability in the PLP10 and the placebo groups, respectively, when patients on natalizumab were excluded. No statistically significant

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7 493 | difference was observed for any comparison of the other two groups ~~compared to~~ with the
8 494 | placebo group (Fig 4A and Supplementary Information Fig 2).

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12 496 | ~~Regarding In~~ the ITT analysis, at two years, the cumulative probability of progression was
13 497 | 10% in the PLP10 group and 35% in the placebo group (p=0.052, a trend for an effect),
14 498 | which represents a decrease of 25 percentage points or a relative 71% decrease ~~of for~~ the
15 499 | PLP10 group with respect to the risk of sustained progression of disability (adjusted
16 500 | ~~hazard ratio 0.22, 95% CI 0.04 to 1.07, p=0.06)~~ (Fig 4B). Two versus seven out of the total
17 501 | randomized patients progressed to confirmed disability in the PLP10 and ~~the~~ placebo groups,
18 502 | respectively. No significant differences were observed for groups A or C ~~against compared~~
19 503 | with the placebo group (Fig 4B). The mean change in ~~Expanded Disability Status Scale (the~~
20 504 | EDSS) score as a function of visit number is shown in Figure 5.

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506 MRI

507 | Over two years, the MRI results supported ~~the overall conclusion from the study that a~~
508 | PLP10 ~~related has a~~ positive effect ~~on disease activity since as~~ only 29% from the PLP10
509 | group, ~~in contrast as opposed~~ to 67% from the placebo group, developed new or enlarging T2
510 | lesions (57% relative risk reduction). ~~After Excluding the~~ patients on natalizumab, there ~~is~~
511 | ~~an~~ was an increased relative risk reduction (64%) ~~between for~~ PLP10 ~~as opposed compared~~
512 | ~~with to the~~ placebo, with 29% of patients on PLP10 and 80% on placebo ~~with~~
513 | ~~developing of~~ new or enlarging T2 lesions (Table 5).

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515 Safety

516 | Over the course of the 30 month study, no significant adverse events were reported ~~from for~~
517 | any group. ~~According to a returned questionnaire questioner procedure t~~The only ~~aetiology~~

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7 518 reasonaetiology for the drop-outs was the palatability and smell of the formula preparations
8
9 519 in addition to pregnancy. Nausea was reported by two patients. No abnormal values were
10
11 520 observed on any of the biochemical and haematological blood tests. No allergic reactions
12
13 521 were reported.
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15 522

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16 523 **Discussion**

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18 524 In this proof-of-concept randomised, double-blind clinical trial assessing the safety and
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20 525 clinical trial assessing the safety and efficacy of three variations of a novel cocktail
21
22 526 nutritional intervention formulas in RRMS, we observed a significant benefit association for
23
24 527 the novel thea formula containing a balanced mixture of specific omega-3 and omega-6
25
26 528 PUFAs, MUFAs, SFAs, vitamin A, vitamin E and γ -tocopherol, (PLP10) intervention
27
28 529 compared to with the placebo for both the ARR and the progression to disability in the per-
29
30 530 protocol analysis. Our results included analyses pertaining to a total of 42 months of study-
31
32 531 collected data, including the 12-month intervention-free treatment extension period. Our
33
34 532 results include analyses pertaining to a total of 42 months study collected data, including the
35
36 533 12 month, free of intervention treatment, extension period. We focused on the per protocol
37
38 534 data analysis since it is the appropriate method to best provide the answer to the proof of
39
40 535 concept trial addressed question. We also observed, aThe high drop-out rate that was mostly
41
42 536 the result of formulas palatability, a common phenomenon in trials using oily interventions
43
44 537 where a lot of patients tend to drop out soon after first dosage. We thus present our main per
45
46 538 protocol analysis, as well as a subgroup analysis excluding patients on natalizumab.
47
48 539 Interestingly, a We have found a statistically significant reduction in the ARR and the
49
50 540 disability progression was also observed when comparing not only patients on PLP10 versus
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52 541 placebo but also comparing the ARR of the PLP10 patients in the 24-month period prior to
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54 542 the study to with the ARR of the 24 months on-study; the observed differences became

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543 larger when patients that received natalizumab (the ~~currently~~ most potent disease modifier)
 544 were excluded. The ARR decreased within a year on PLP10 and ~~significantly~~ remained stable
 545 until ~~the~~ study completion. ~~The S~~statistically significant ~~in the~~ difference of ARR between
 546 patients on PLP10 ~~versus and those on~~ placebo continued for the ~~additional~~ 12 month
 547 ~~extended period (persistent effect) without a significant difference on the DMT after the~~
 548 ~~study extended period (persistent effect) without significant differences on DMT~~. These
 549 clinical findings are supported by the results ~~regarding from~~ the MRI analysis ~~where in which~~
 550 the proportion of patients free from new or enlarging brain T2 lesions was also higher in ~~the~~
 551 ~~PLP10 group versus than the~~ placebo ~~group~~. The persistent effect within the extended period
 552 ~~it is considered believed to be~~ of major importance and supportive of the results since it is in
 553 ~~agreement with the very long washouts, reported necessary, for omega 3 fatty acids and~~
 554 ~~especially DHA to return towards pretreatment values within the fatty acids of plasma,~~
 555 ~~platelets, monocytes and red blood cells.~~⁴² This study also provides important 30 month,
 556 ~~placebo controlled information about the safety of PLP10, A and C interventions.~~ No severe
 557 side effects have been reported.

558

559 ~~As medications used to treat MS become increasingly highly specific and potent, attention to~~
 560 ~~safety is paramount. Current available treatments are products of reductionism, partially~~
 561 ~~effective, associated with severe side effects without (re)myelinating or neuroprotective~~
 562 ~~abilities.~~

563 ~~To the best of our knowledge, this study is the first randomized clinical trial assessing the~~
 564 proposed combination of active ingredients in a standardized proportion and dosing scheme for
 565 MS treatment designed according to the systems medicine approach. Nutrition is commonly
 566 accepted as one of the possible environmental factors involved in the pathogenesis of MS, but
 567 its role as a complementary MS treatment is unclear and largely disregarded.⁵¹ It is well

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7 568 known that the majority of the patients suffering from MS do use dietary supplements for a
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9 569 variable length of time.⁵² Dietary antioxidants and fatty acids may influence the disease
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11 570 process in MS by reducing immune-mediated inflammation, oxidative stress and excitotoxic
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13 571 damage.¹² Published data have revealed that healthy dietary molecules have a pleiotropic role
14
15 572 and are able to change cell metabolism and down-regulate inflammation by interacting with
16
17 573 enzymes, nuclear receptors and transcriptional factors.⁵¹ Current available treatments are the
18
19 574 products of reductionism, partially effective and associated with severe side effects.
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21 575 Interferons and glatiramer acetate, the most widely used first-line MS drugs available today,
22
23 576 are associated with the least severe side effects among the MS therapies, but they are reported
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25 577 to reduce the ARR only by about one third and with no significant effect on the progression
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27 578 of disability.⁵³ Natalizumab reduces the ARR by 68% and decreases the possibility of
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29 579 disability progression by 43%, with 57% of patients free of new or enlarging T2 lesions on
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31 580 MRI scans, compared with 15% on placebo.⁵⁴ Fingolimod is associated with a 54% ARR
32
33 581 reduction (without a significant benefit on the progression of disability). Both natalizumab
34
35 582 and fingolimod are second-line drugs associated with severe side-effects.⁵⁵
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37 583 ~~efficacy of a~~ After a thorough search in the literature we are convinced that no existing MS
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39 584 ~~treatment module has ever been designed according to as a result of the SM systems~~
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41 585 ~~medicine concept approach, or with a potential to effectively stimulate intrinsic~~
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43 586 ~~remyelinating and neuroprotecting mechanisms or exert such an action.~~ Now w
44
45 587 Mehta in a review paper, in 2009, reported different clinical studies on interventions
46
47 588 formulated based on the individual aforementioned molecular ingredients or based on a
48
49 589 specific ratio of the aforementioned molecular ingredients for MS treatment; although no one
50
51 590 was reported using the antioxidant vitamin γ -tocopherol.⁵⁶ In our study, the choice of
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53 591 ingredient proportion and dosing scheme was based upon evidence derived form *in vivo* and
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55 592 *in vitro* data. In the Western diet, the ratio of omega-3 to omega-6 is about 1:20–30; in

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7 593 populations that consume fish-based diets, the ratio is about 1:1–2.^{52,53} The intervention daily
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9 594 dose was designed aiming and believed to be high enough to restore/amplify body efficient
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11 595 antioxidant activity and ensure cellular membranes lipid profile normalization (PUFA
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13 596 content) and simultaneously potentiate involvement of the ingredients in the anti-
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15 597 inflammatory and recovery mechanisms. Diet fatty acid molecules need about a six months
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17 598 period to exert their beneficial effect and this essential parameter was for the first time under
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19 599 consideration in our study design (normalization period).⁴² This chronotherapy parameter
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21 600 might be of major importance and is in line with the systems medicine treatment philosophy.
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23 601 We believe that the persistent effect within the post-study period is in agreement with the
24
25 602 reported very long washout phase for omega-3 fatty acids, especially DHA, to return to the
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27 603 pre-treatment values.⁴⁶ Considering that omega-3 supplementation can release and replace
28
29 604 excess AA within the cellular membranes, we can speculate that an increased inflammatory
30
31 605 activity can possibly result during the first six months of supplementation.
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34 607 In addition to the EPA, DHA, LA, and GLA, PLP10 contained limited quantities of other
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36 608 structural/active PUFAs, specific MUFAs (mostly oleic acid) and SFAs (palmitic and stearic
37
38 609 acid), specifically to provide a direct source for neuronal cell membranes rehabilitation and
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40 610 for (re)myelination and neuroprotection because these compounds are all major components,
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42 611 precursors and building blocks of any new physiological myelin and cellular membranes in
43
44 612 general. Assembly of the correct molecules into the myelin membrane may be especially
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46 613 critical during active synthesis. If these critical constituents aren't directly or indirectly
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48 614 available, amyelination, dysmyelination or demyelination may ensue.⁵⁶ The maintenance of
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50 615 myelin requires continued turnover of its components throughout life.^{54,55}
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7 617 Different factors and molecular entities appear to be part of the possible aetiology for MS,
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9 618 with specific PUFA and antioxidants found to be key substances related to all known
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11 619 pathogenic and recovery mechanisms. In our study, we further proposed that a holistic
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13 620 systems medicine model approach can be applied by synchronized action. First, there is an
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15 621 obvious convenience in administering one formula containing different specific active
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17 622 ingredients. The currently available evidence supports that nutritional interventions would
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19 623 confer a small to medium treatment effect with an accompanying appropriate safety profile.¹²
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21 624 ^{52 56} Combining these specific active ingredients together with γ -tocopherol and other specific
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23 625 active molecules into one stable formulation is expected to enhance adherence while still
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25 626 offering an appropriate safety profile. A similar approach could not be adopted for
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27 627 pharmaceutical interventions with common and severe adverse events, such as those
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29 628 indicated today for patients with MS. Given the advantages of the simultaneous use and that
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31 629 all the included ingredients have proven individually a valid biological plausibility and have
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33 630 been tested in various settings and under various dose schemes, we also assessed the
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35 631 hypothesis that a novel mixture of these ingredients would have a postulated efficacy attained
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37 632 synergistically through different mechanisms of action.^{52 56} Interestingly, the observed
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39 633 magnitude of the treatment effect cannot be explained by adding up the postulated efficacy
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41 634 estimates of the individual ingredients. Findings from *in vitro* and *in vivo* studies support this
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43 635 notion of proposed synergy although this hypothesis can only be taken forward when the
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45 636 observed treatment effect is validated in various settings and in a larger number of patients.
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49 638 We acknowledge that our study has two considerable limitations: the small sample size and
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51 639 the high drop-out rate. Regarding the sample size, one should bear in mind that this study is a
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53 640 small, phase-2 clinical trial assessing a novel intervention and thus has comparable size in the
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55 641 appropriate literature. Questions taken forward from this trial can be assessed in a larger

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7 642 [randomised trial in which appropriate power calculations would be possible, taking into](#)
8 643 [consideration the findings of the present study. The adherence of the subjects is another](#)
9 644 [limitation of our study, but the total duration of the study that covers a total of 42 months](#)
10 645 [follow-up adds power to the results.⁴⁸ We acknowledge that we had to deliver the](#)
11 646 [intervention in the way most frequently associated with low compliance, i.e., an oral, liquid](#)
12 647 [formula, thus triggering maximum intolerance due to taste. Nevertheless, the observed](#)
13 648 [suboptimal compliance is in accordance with the published literature in which clinical trials](#)
14 649 [assessing liquid fatty acid interventions show a weaker adherence compared with clinical](#)
15 650 [trials of pharmaceutical interventions. Indeed, in our study, we consistently recorded the](#)
16 651 [reasons for withdrawal: most of the participants did not discontinue due to safety issues, but](#)
17 652 [rather due to palatability issues. Controlling non-compliance due to palatability issues is by](#)
18 653 [far easier to address compared with non-compliance related to adverse events and can be](#)
19 654 [resolved when optimisation of the formulation is achieved in future trials. At this stage of the](#)
20 655 [development of the intervention, we would by far exceed the cost-effectiveness threshold if](#)
21 656 [we were to invest in improving these features of the intervention. Moreover, we should also](#)
22 657 [note that MS patients are subject to far more frequent and more serious adverse events related](#)
23 658 [to the current standard treatments.](#)

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25 660 [As a direct consequence of the low compliance and the loss of power, the performed](#)
26 661 [intention-to-treat analysis was far less robust than intended, and we would then have to take](#)
27 662 [into serious consideration the performed per-protocol analysis. We focused on the per-](#)
28 663 [protocol data analysis because it is the appropriate method to best provide the answer for the](#)
29 664 [proof-of-concept trial-addressed question.²⁴ To validly incorporate the results of the per-](#)
30 665 [protocol analysis into the interpretation of the overall results of the trial, we needed to ensure](#)
31 666 [that the randomisation was not seriously violated due to the exclusion of the non-compliers.](#)

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7 667 The comparison between the baseline characteristics of the patients included in the per-
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9 668 protocol analysis did show a relative balance in the compared groups for known confounders.
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11 669 Nevertheless, the presence of unknown confounders introducing bias to the trial results
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13 670 cannot be excluded despite non- significant differences in the baseline characteristics. As an
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15 671 additional safeguard towards that end, we also performed adjusted analyses for the primary
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17 672 and secondary analyses for important clinical and demographic parameters, i.e., relapses,
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19 673 EDSS, age and DMT.

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22 675 We proposed that a holistic SM model approach has to be applied by synchronized action on
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24 676 all the involved perturbed mechanisms. Although, all the included ingredients have proven
25
26 677 individually a valid biological plausibility and have been tested in various settings and under
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28 678 various dose schemes (REFS HERE), we assessed the hypothesis that a novel mixture of this
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30 679 ingredients PLP10 has a innovative characteristics with a ppostulated efficacy attained
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32 680 through different mechanisms of action and probably by the synergistic effect of its
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34 681 constituent ingredients. Reasons thereof other than the obvious convenience of administering
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36 682 one formula containing different active ingredients, are . Moreover, the currently available
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38 683 evidence supports that nutritional interventions would confer a small to medium treatment
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40 684 effect (ref). The notion of combining these interventions into one stable formulation would be
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42 685 expected to provide a maximum adherence with a appropriate safety profile. An similar
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44 686 approach could not be adopted for pharmaceutical interventions with common and severe
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46 687 adverse events such as these indicated today for the MS patients., PLP10 has all the
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48 688 characteristics of a medical food with the action to feed a normal metabolic process by
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50 689 supplying nutritional structural membrane precursors, building blocks, and vitamins from
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52 690 dietary sources that enhance remyelination and neuroprotection and simultaneously promote

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7 691 normalization of all cellular membranes lipid content. The intention is to normalize the
8 692 specific nutritional requirements of the MS patients.
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12 694 Interestingly, in this small phase II trial, we observed a far larger treatment effect than
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14 695 expected. One explanation could be maximum synergistic effect observed when
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16 696 simultaneously administering the assessed ingredients. Findings from in vitro and in vivo
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18 697 studies could support this notion. Nevertheless, this hypothesis can only be taken forward
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20 698 when Different factors and molecular entities appear to be part of the possible aetiology for
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22 699 MS with specific PUFA and antioxidants found to be key substances related to all known
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24 700 pathogenic and recovery mechanisms. But, it is well established that MS patients are
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26 701 characterized with significant deficiencies in antioxidant efficiency as well as in PUFA levels
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28 702 in blood and cellular membranes.^{41, 49-51}
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32 704 According to one hypothesis, the change in the ratio of omega 3 to omega 6, due to the
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34 705 increasing consumption of omega 6 PUFA and resulting in, meaning high accumulation of
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36 706 AA, in the Western diet, may be one of the major factors responsible for the increasing
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38 707 incidence of inflammatory diseases relative to populations. In the Western diet, the ratio of
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40 708 omega 3 to omega 6 is about 1:20-30; in populations that consume fish-based diets, the ratio
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42 709 is about 1:1-2.⁵²⁻⁵³ The intervention daily dose was designed aiming and believed to be high
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44 710 enough to restore/amplify body efficient antioxidant activity and ensure cellular membranes
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46 711 lipid profile normalization (PUFA content) and simultaneously potentiate involvement of the
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48 712 ingredients in the anti-inflammatory and recovery mechanisms. Diet fatty acid molecules
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50 713 need about a six months period to exert their beneficial effect and this essential parameter
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52 714 was for the first time under consideration in our study design (normalization period).⁴² This
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54 715 chronotherapy parameter it is of major importance in line with the SM treatment philosophy

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7 716 We believe that the persistent effect within the post study period is in agreement with the
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9 717 reported very long washout phase for omega 3 fatty acids, especially DHA, to return to the
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11 718 pre treatment values.⁴⁶ and if it is not included in the trial design the possibility of misleading
12
13 719 result evaluation greatly increases. In fact, considering that omega 3 supplementation can
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15 720 release and replace excess AA within the cellular membranes, we can speculate that an
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17 721 increased inflammatory activity can possibly result during the first six months of
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19 722 supplementation (during normalization period).
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22 724 The maintenance of myelin requires continued turnover of its components throughout life.^{54,55}
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24 725 In addition to the EPA, DHA, LA, and GLA, PLP10 was containing limited quantities of
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26 726 other structural PUFA, specific MUFA (mostly oleic acid) and SFA (palmitic and stearic
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28 727 acid), specifically aiming to provide a direct source for neuronal cell membranes
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30 728 rehabilitation and for (re)myelination and neuroprotection since they are all major
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32 729 components, precursors and building blocks of any new physiological myelin and cellular
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34 730 membranes in general. Assembly of the correct molecules into myelin membrane may be
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36 731 especially critical during active synthesis. Possibly, if critical constituents aren't available or
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38 732 are metabolically blocked, amyelination, dysmyelination or demyelination may ensue.⁵⁶
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41 734 We acknowledge that our study has two considerable limitations pertaining to the small
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43 735 sample size and the high drop out rate. Regarding the sample size, one has to bear in mind
44
45 736 that this is a small, phase 2 clinical trial assessing a novel intervention and has thus
46
47 737 comparable size in the relative literature. Questions taken forward from this trial can be
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49 738 assessed in a larger randomized trial where appropriate power calculations would be possible
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51 739 taking into consideration the findings of the present study. The adherence of the subjects is
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53 740 another limitation of our study since almost half of the participants withdrew. We
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7 741 acknowledge that we had to deliver the intervention in the way most frequently associated
8 742 with low compliance, i.e. oral, liquid formula, thus triggering maximum intolerance due to
9 743 taste. Nevertheless, the observed suboptimal compliance is in accordance with the published
10 744 literature where clinical trials assessing liquid fatty acid interventions show a weaker
11 745 adherence compared to clinical trials of pharmaceutical interventions. Indeed, in our study,
12 746 we consistently recorded the reasons of withdrawal and most of the participants did not
13 747 discontinue due to safety issues, but mostly in relation to palatability issues. Controlling non-
14 748 compliance due to palatability issues is by far easier to deal with compared to non-
15 749 compliance related to adverse events and can be resolved when optimization of the
16 750 formulation is achieved in future trials. At this stage of the development of the intervention,
17 751 we would by far exceed the cost effectiveness threshold if we were to invest in improving
18 752 these features of the intervention. Moreover, we should also note that MS patients are subject
19 753 to far more frequent and more serious adverse events related to the current standard
20 754 treatment.

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35 756 As a direct consequence of the low compliance and the loss of power, the performed
36 757 intention to treat analysis was far less robust than intended and we would then have to take
37 758 into serious consideration the performed pre-protocol analysis. In order to validly incorporate
38 759 the results of the pre-protocol analysis into the interpretation of the overall results of the trial,
39 760 we needed to ensure that the randomization was not seriously violated due to the exclusion of
40 761 the non-compliers. The comparison between the baseline characteristics of the patients
41 762 included in the per protocol analysis did show a relative balance in the compared groups for
42 763 known confounders. Nevertheless, the presence of unknown confounders introducing bias in
43 764 the trial results cannot be excluded despite non-statistically significant differences in the
44 765 baseline characteristics. As an additional safeguard towards that end, we also performed

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7 766 ~~adjusted analyses for the primary and secondary analyses for important clinical and~~
8 ~~demographic parameters i.e. relapses, EDSS, age and DMT.~~

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12 769 ~~The present preliminary, small size, randomized, controlled phase II clinical trial provides~~
13 ~~evidence for a novel nutritional/nutraceutical formula based on dietary, metabolic,~~
14 770 ~~immunological, and neurobiological pathways possibly involved with disease progression in~~
15 771 ~~MS. This novel intervention showed signs of efficacy in the observed annualised relapse rates~~
16 772 ~~and disability progression to disability. -We took the appropriate all methodological~~
17 773 ~~precautions measures in order to control for potential sources of bias and be to enable able to~~
18 774 ~~reach a valid interpretation to be reached. We acknowledge that the presence of bias can only~~
19 775 ~~be minimized, yet not excluded, in any clinical research setting and also that random error is~~
20 776 ~~always a possible scenario in small trials. Thus, we present the observed results as an~~
21 777 ~~additional piece of randomized evidence and anticipate the replication of our study findings~~
22 778 ~~in a larger randomised controlled clinical trial.~~

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26 781 ~~The well known and established safety of the ingredients used and the protocol guidelines~~
27 782 ~~were supportive reasons for us to proceed with the clinical study even though with limitation~~
28 783 ~~on the pre-estimation of required trial sample size as it was discussed in method section. The~~
29 784 ~~adherence of the subjects is Interferons and glatiramer acetate, the most widely used first line~~
30 785 ~~MS drugs available today, are associated with the least severe side effects among MS~~
31 786 ~~therapies but they are reported with only 29-33% ARR reduction and with no significant~~
32 787 ~~effects on the progression of disability. Natalizumab as previously discussed and Fingolimod~~
33 788 ~~with 54% ARR reduction (without significant benefit on the progression of disability) are~~
34 789 ~~second line drugs associated with severe side effects.^{47,48}~~

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7 790 ~~another issue but the duration of the study (42 months) is adding power to the results;⁴⁴~~
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9 791 ~~having the research questions been consciously and carefully approached and answered.~~
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11 792 ~~Furthermore, the statistical methodologies used along with the appropriate adjustments,~~
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13 793 ~~broadly accepted for MS clinical trials, power strengthen the findings, results, and~~
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15 794 ~~significance. The baseline characteristics of the treatment arms could possibly be considered~~
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17 795 ~~indicative of four very active groups of patients but that was the result of the limited number~~
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19 796 ~~of RRMS population eligible for the study within Cyprus. On the other hand the balanced~~
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21 797 ~~baseline characteristics without statistical differences, the statistical adjustments (for all~~
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23 798 ~~important baseline parameters i.e. relapses, EDSS, age and DMT) and the randomization~~
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25 799 ~~within four different groups are the safety valves against data misinterpretation. Yet, in small~~
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27 800 ~~randomized control trials with a high drop out rate, the per protocol analysis could be~~
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29 801 ~~affected by the characteristics of the patients dropping out. In order to safeguard our findings~~
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31 802 ~~in the best possible way under the circumstances, we proceeded to adjusting for confounders.~~
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33 803 ~~Moreover, we cannot discard our finding as a false positive, given that this is a randomized,~~
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35 804 ~~double blind, placebo controlled clinical trial and, despite its small sample size, represents a~~
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37 805 ~~piece of evidence that only a larger randomized controlled trial can replicate or refute. It is~~
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39 806 ~~possible to question why DMTs efficacy cannot be emerged out of the data analysis, of the~~
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41 807 ~~four treatment arms, and in accordance to their published values. We believe that the limited~~
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43 808 ~~efficacy of the DMTs, the sample size and the statistical adjustments were strong limiting~~
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45 809 ~~determining factors for such an indication to be countable. An additional argument is that the~~
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47 810 ~~efficacy reported for the analysis of pre treatment (24 months before entry baseline) versus~~
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49 811 ~~on trial ARR could be considered as potentially biased due to differences of how relapses~~
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51 812 ~~were defined during the course of a study compared to pre treatment period; or due to~~
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53 813 ~~regression to the mean or placebo effect. This analysis was performed as an additional~~
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55 814 ~~exploratory analysis that we were able to do due to the availability of data. The relapses of~~

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7 815 the two pre-treatment years were drawn out of the patients' archival records by the same
8 816 treating neurologist involved in the study (MP), and according to the patients' hospitalization
9 817 date for receiving intravenous methyl prednisolone. This analysis was not used as a primary
10 818 or a secondary end-point under investigation although it is usually reported by many clinical
11 819 studies. As a matter of fact many early phase trials are based only on such an analysis (before
12 820 versus after treatment results). In almost all MS trials the number of relapses within the two
13 821 years before baseline is a factor under adjustment for the statistical analyses.⁴⁸ The inclusion
14 822 of the post hoc MRI analysis is another limiting factor that needs attention since it was used
15 823 as an additional aside exploratory approach (due to study budget limitations it was not
16 824 possible to be used as a formal endpoint); but the MRI evaluation was blinded and can be
17 825 considered as representative of the randomized subjects within the treatment arms. As far as
18 826 the regression to the mean and the placebo effect concerns we believe that the 6-month
19 827 normalization period is an accountable and valuable eliminating factor of the possible effect;
20 828 as well as the presence of four groups, where only the PLP10 treatment arm is associated
21 829 with statistically significant efficacy versus placebo.

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23 831 Our observations are consistent with the idea that simultaneous availability of specific PUFA
24 832 along with other major membrane and myelin building blocks in combination with specific
25 833 antioxidants, within optimum quantity, quality, ratio and structural form can possibly result to
26 834 a more appropriate holistic therapy reducing MS disease activity. It is our belief that this is
27 835 probably succeeded through synergistic and/or simultaneous effect on the interactions and
28 836 dynamics of the most probable environmental and biological disease causing factors that
29 837 induce complex biological network of events for disease pathogenesis and evolution; as well
30 838 as on the protective and reparative mechanisms. We can additionally speculate that the nature
31 839 of the intervention formula cannot be prohibitive for its use as preventive regimen and does

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7 840 not preclude probable positive efficacy on the other types of MS, but has to be further
8 841 investigated. A larger size multicenter clinical trial will better establish PLP10 place in the
9 842 armamentarium of treatments for MS.

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10 844 It is commonly accepted that nutrition is one of the possible environmental factors involved
11 845 in the pathogenesis of MS, but its role as complementary MS treatment is unclear and largely
12 846 disregarded.⁵⁷ It is well known that the majority of the patients suffering from MS they do
13 847 use dietary supplements for a variable length of time and they prefer supplement type of
14 848 "help" over conventional drugs.⁵⁸ Dietary antioxidants and fatty acids may influence the
15 849 disease process in MS by reducing immune-mediated inflammation, oxidative stress and
16 850 excitotoxic damage.¹¹ Present data reveal that healthy dietary molecules have a pleiotropic
17 851 role and are able to change cell metabolism from anabolism to catabolism and down-regulate
18 852 inflammation by interacting with enzymes, nuclear receptors and transcriptional factors.⁵⁷
19 853 The present preliminary small size randomized controlled phase II clinical trial, for the first
20 854 time provides link evidence between dietary, metabolic, immunological, and neurobiological
21 855 aspects of MS after three quarters of a century of unsuccessful scientific efforts. This link
22 856 evidence might probably be the beginning of opening new horizons and new avenues in the
23 857 approach of MS prevention and treatment, and possibly of other multifactorial chronic
24 858 diseases, including neurodegenerative and autoimmune as well.

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Treatment Arms			
A†	B (PLP10)†	C†	Placebo†
Intervention: EPA (1,650mg) / DHA (4,650mg) / GLA (2,000mg) / LA (3,850mg) / total other omega-3 (600mg)* / total MUFA** (1,714mg) + total SFA (18:0 160mg, 16:0 650mg) / vitamin A (0.6mg) / vitamin E (22mg) plus citrus aroma	Intervention: EPA (1,650mg) / DHA (4,650mg) / GLA (2,000mg) / LA (3,850mg) / total other omega-3 (600mg)* / total MUFA** (1,714mg) + total SFA (18:0 160mg, 16:0 650mg) / vitamin A (0.6mg) / vitamin E (22mg) + pure γ -tocopherol (760mg) plus citrus aroma	Intervention: pure γ -tocopherol (760mg) dispersed in pure virgin olive oil (16,137 mg) as delivery vehicle plus citrus aroma	Intervention: Olive oil (pure virgin) plus citrus aroma
* Other omega-3: C18:3n-3 37mg, C18:4n-3 73mg, C20:4n-3 98mg, C22:5n-3 392mg			
** MUFA: 18:1 1300mg, 20:1 250mg, 22:1 82mg, 24:1 82mg			
† Total daily dose 19.5ml			
EPAX1050, EPAX AS, Aalesund, Norway; was used as the source for the omega-3 PUFA, as re-esterified glycerides from fish body oils; Borage seed oil (organic, cold pressed) "Borago officinalis" Goerlich Pharma International GmbH, Edling, Germany, was used as the source for the omega-6 PUFA, MUFA and SFA, as triglycerides. The pure γ -tocopherol was purchased from Tama Biochemical Co. Ltd., Shinjuku-ku Tokyo, Japan; vitamin A as beta-carotene from HealthAid Ltd., Middlesex, United Kingdom and the Citrus aroma from Givaudan Schwaiz AG, Dubendorf, Switzerland.			

Table 1. Intervention ingredients per treatment arm.

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2A.					
Characteristics	Group A (n=20)	Group B† (n=20)	Group C (n=20)	Placebo (n=20)	P- value
Sex					
Female - no. (%)	15 (75)	15 (75)	15 (75)	15 (75)	1.000
Age (yr)					
Mean ± Standard Deviation (SD)	38.0±11.9	36.9±8.4	37.7±8.7	38.1±10.9	0.982
Median (Range)	38.0 (22 – 65)	37.0 (25 – 61)	36.5 (24 – 54)	36.0 (21–58)	
Treatment history					
Patients on DMT- no. (%)	11 (55)	9 (45)	12 (60)	10 (50)	0.875
Pre-treatment disease duration (yr)					
Mean ± SD	9.0±7.6	8.6±4.8	8.6±5.3	7.7±5.7	0.909
Median (Range)	7.5 (2 – 37)	8.0 (2 – 20)	8.0 (3 – 24)	6.5 (2 – 25)	
Pre-treatment relapses‡					
Mean ± SD	2.33±1.68	2.41±1.73	2.31±1.66	2.10±1.32	0.946
Median (Range)	2.0 (1 – 6)	2.0 (1 – 7)	2.0 (1 – 6)	2.0 (1 – 4)	
ARR	1.17	1.21	1.16	1.05	0.946
Patients -% with ≤1 relapse	40	45	40	35	

Baseline EDSS score‡					
Mean ± SD	2.52±1.23	2.15±1.05	2.42±1.21	2.39± 0.93	0.775
Median (Range)	2.5 (1.0–5.5)	2.0 (1.0–4.0)	2.5 (0.0–5.0)	2.5 (1.0–4.0)	
2B.					
Characteristics	Group A (n=10)	Group B† (n=10)	Group C (n=9)	Placebo (n=12)	P- value
Sex					
Female - no. (%)	5 (50)	7 (70)	6 (66.6)	10 (83.3)	0.419
Age (yr)					
Mean ± SD	36.6±13.5	34.80±5.4	40.9±8.1	39.8±13.2	0.572
Median (Range)	34.5 (22–65)	34.5 (26–43)	40.0 (29–54)	37.5 (21–58)	
Treatment history					
Patients on DMT- no. (%)	6 (60)	4 (40)	6 (67)	6 (50)	0.949
Pre-treatment disease duration (yr)					
Mean ± SD	9.7±10.0	8.3±5.3	11.3±6.1	8.7± 7.1	0.807
Median (Range)	7.5 (2 – 37)	8.0 (2 – 20)	8.0 (4 – 24)	5.5 (2 – 25)	
Pre-treatment relapses					
Mean ± SD	2.20±1.47	2.70±1.25	1.78±0.66	1.67±1.37	0.241
Median (Range)	2.0 (1 – 6)	2.5 (1 – 4)	2.0 (1 – 3)	1.5 (1 – 4)	
ARR	1.10	1.35	0.89	0.83	
Patients -% with ≤1 relapse	30	20	33	50	
Baseline EDSS score					
Mean ± SD	2.65±1.37	2.40±1.12	2.11±1.02	2.16±0.96	0.698
Median (Range)	3.0 (1.0–5.5)	2.5 (1.0–4.0)	2.0 (1.0–4.0)	2.0 (1.0–3.5)	

† PLP10 group

‡ Available data at Entry Baseline (n=18 for group A, n=17 for group B, n=19 for group C, n=19 for group D)

Table 2. The table section 2A reports the demographics and baseline disease characteristics for total randomized population by treatment arm.

The table section 2B reports the demographics and baseline disease characteristics of all-time on-study population by treatment arm. There were no significant between study-group differences at baseline for any characteristic.

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3A.									
Characteristics	Group A (N=10)		Group B† (N=10)		Group C (N=9)		Placebo (N=12)		
End Point	X	Y	X	Y	X	Y	X	Y	
Total No. of relapses	22	17	27	8	16	13	20	25	
Annual Relapse Rate (ARR)	1.10	0.85	1.35	0.40	0.88	0.72	0.83	1.04	
% Reduction Compared to Placebo (primary endpoint) (Ys)¶		-18		-62		-30			N/A
P Value against placebo		0.468		0.024		0.578			
ARR change -% (Y to X)¶		-23		-70		-18			+25
P value against baseline		0.425		0.003		0.578			0.500
X: Total number of relapses of 24 months pre-treatment (baseline)									
Y: Total number of relapses of 24 months on-treatment									
¶ Unadjusted estimate									
3B.									
<u>Excluding patients on natalizumab</u>	Group A (N=9)		Group B† (N=10)		Group C (N=9)		Placebo (N=10)		

End Point	X	Y	X	Y	X	Y	X	Y
Total No. of relapses	16	15	27	8	16	13	13	19
Annual Relapse Rate (ARR)	0.88	0.83	1.35	0.40	0.88	0.72	0.65	0.95
% Reduction Compared to Placebo (primary endpoint) (Ys)¶		-13		-58		-24		N/A
P Value against placebo		0.493		0.016		0.412		
ARR change -% (Y to X)¶		-6		-70		-18		+46
P value against baseline		0.857		0.003		0.578		0.354

X: Total number of relapses of 24 months pre-treatment (baseline)
 Y: Total number of relapses of 24 months on-treatment
 † PLP10 group
 ¶ Unadjusted estimate

Table 3. The table section 3A reports the two year primary end points of ARR of all-time on-study population by treatment arm and percent difference with placebo. During the 24mo period on-treatment the ARR of the group A was 0.85 with 18% decrease compared to placebo (p=0.468), of PLP10 group 0.40 with 62% decrease (p=0.024), and of group C 0.72 with 30% decrease (p=0.578) and reports the comparison of the 24mo pre-treatment ARR (baseline ARR) to 24mo on-treatment ARR of all-time on-study population including patients on natalizumab.

The table section 3B reports comparison of the 24mo pre-treatment ARR to 24mo on-treatment ARR of all-time on-study population excluding patients on natalizumab; and the comparison of the ARR during the 24mo period on-treatment (primary end point) between each one of the groups against placebo.

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4A.								
Characteristics	Group A (N =8)		Group B† (N =7)		Group C (N =10)		Placebo (N =7)	
	X	Y	X	Y	X	Y	X	Y
No. of Relapses	20	14	14	14	27	26	20	13
Annual Relapse Rate (ARR)	1.25	0.88	1.00	1.00	1.35	1.30	1.42	0.92
X: Total number of relapses of 24 months pre-treatment Y: Total number of relapses of 24 months on-treatment								
4B.								
Characteristics	Group A (N =20)		Group B† (N =20)		Group C (N =20)		Placebo (N =20)	
End Point	X	Y	X	Y	X	Y	X	Y
No. of Relapses	45	34	49	30	46	41	43	41
Annual Relapse Rate (ARR)	1.13	0.85	1.23	0.75	1.15	1.03	1.08	1.03
ARR Reduction -% (Y to X)¶	-25		-39		-10		-5	
P value against baseline	0.120		0.005		0.475		0.652	
% Reduction of the ARR Compared to Placebo (Ys)¶	-18		-27		0.0		N/A	
P Value against placebo	0.447		0.121		0.996			

X: Total number of relapses of 24 months pre-treatment (baseline)
 Y: Total number of relapses of 24 months on-treatment
 ¶ Unadjusted estimate
 † PLP10 group

Table 4. The table section 4A reports the two year primary end point of relapses based on study design as reported by drop-out patients by Treatment Arm. The most drop-out patients that went on disease modified therapy (DMT) were from the group A and placebo with three and two patients respectively on natalizumab. These parameters justify the decreased number of relapses recorded within group A and placebo drop-outs; and this could affect the ITT analysis in favor of placebo when the total two-year recorded data are used. For PLP10 group, 14 relapses were reported at baseline and remained the same during the two-year study. For placebo, 20 relapses were reported at baseline and decreased to 13 during the two-year study. This is expected since, for PLP10 group 43% of the drop-outs were under DMT at entry baseline and remained the same until the end of the study with no patient on natalizumab; but 57% of the placebo group drop-outs that were under DMT at entry baseline increased to 86% at the end of the study including two patients on natalizumab.

The table section 4B reports comparison of 24 months pre-treatment ARR (baseline) to 24 months on-treatment ARR of total randomized population, by treatment arm. The ARR of PLP10 group was 1.23 at baseline and 0.75 at the end of the study (39% reduction p=0.005), and for placebo 1.08 at baseline and 1.03 at the end of the study (5% reduction p=0.652). No statistical difference was calculated for the other two treatment arms. During the 24 months on-treatment, PLP10 group presented a 27% reduction of ARR versus placebo (p=0.121), with all groups without statistically significant results.

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5.					
Characteristics*	Group A (n=10)	Group B PLP10 (n=10)	Group C (n=9)	Placebo (n=12)	P-value Group B vs. Placebo
Annual relapse rate over 1 year**	0.80	0.40	0.78	0.83	
Total number of relapses**	8	4	7	10	
Primary end points					
Annual relapse rate over 2 years (95% CI)**	0.85	0.40 (0.15-0.87)	0.72	1.04	0.024
Total number of relapses**	17	8	13	25	
Excluding patients on natalizumab					
	(n=9)	(n=10)	(n=9)	(n=10)	
Annual relapse rate over 2 years (95% CI)	0.83	0.40 (0.10-0.79)	0.72	0.95	0.016
Total number of relapses	15	8	13	19	
Secondary end points					
Cumulative probability of sustained progression increase by 1 point on EDSS confirmed after 6 mo, over 2 years -% **	43	10 (1/10)	24	58 (7/12)	0.019
Excluding patients on natalizumab					
cumulative probability of sustained progression increase by 1 point on EDSS confirmed after 6 mo, over 2 years -%	33	10 (1/10)	24	70 (7/10)	0.006

Exploratory Results					
Patients proportion with ≤ 1 relapse over 2 years -% **	50 (5/10)	90 (9/10)	56 (5/9)	42 (5/12)	0.030
MRI					
Patients proportion with new or enlarging T2 lesions-% **	----	29 (2/7)	----	67 (4/6)	
Excluding patients on natalizumab					
Patients proportion with no new or enlarging T2 lesions-%	----	29 (2/7)	----	80 (4/5)	
DMT (interferons, glatiramer acetate) and natalizumab					
Patients proportion on DMT and natalizumab at the end of 2 years-% **	80 (8/10)†	60 (6/10)	67 (6/9)	75 (9/12) ‡	0.747
* CI denotes confidence interval.					
** Including patients on natalizumab					
† 1 out of 10 on natalizumab					
‡ 2 out of 12 on natalizumab					

Table 5. Clinical end points, according to study group for all-time on-study population.

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Contributors: All authors interpreted the data. I.S.P drafted the report and figures and all authors critically revised and approved the final version. M.C.P and I.S.P were responsible

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7 943 for the protocol and study design. M.C.P was the evaluator of patients' clinical progress signs
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9 944 and the treatment physician. I.S.P and G.N.L were involved in the non-pharmacologic
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11 945 treatment-related care. I.S.P performed the literature search and both I.S.P and G.N.L
12
13 946 contributed on the intervention formulation and composition rational. I.S.P supervised the
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15 947 composition procedure of the interventions and the fatty acid profile analysis of the red blood
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17 948 cell membranes. E.E.N and I.S.P performed the statistical analyses. E.E.N was an
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19 949 independent scientist. All authors vouch for the accuracy and completeness of the data and
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21 950 the statistical analyses.

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26 953 Tourism, program for the creation of new high technology and innovation enterprises through
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28 954 the business incubator.

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45 963 No pharmaceutical companies were involved in this phase II clinical trial. The intervention is
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47 964 under a USA provisional patent; Application Number 61469081.

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51 966 Ethical approval: Cyprus National bioethics committee (reference EEBK/EII/2005/10),
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7 968 All authors have completed the Unified Competing Interest form at
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9 969 www.icmje.org/coi_disclosure.pdf (available on request from the corresponding author) and
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19 974 Ltd. that might have an interest in the submitted work in the previous 3 years; (3) their
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21 975 spouses, partners, or children have no financial relationships that may be relevant to the
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23 976 submitted work; and (4) E.E.N has a non-financial interests that may be relevant to the
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Article Summary

Article focus:

- The increasing prevalence of Multiple Sclerosis (MS) combined with the limited efficacy and the side effects of the existing treatments urge the clinical need for the development of new, innovative, more effective, safe, and preventive treatment strategies.

- ~~For the first time w~~We propose three novel nutraceutical treatment interventions,~~original nutraceutical treatment intervention cocktails~~, formulated based on systems medicine rational through nutritional systems biology~~-rational~~; with PLP10 representing the complete composition of the formulation and the other two treatments represent partial formulations.
- We have studied the proposed interventions on 80 relapsing remitting MS patients (four groups of 20 each) for a total of 42 months (including the 12-month extended period), in a randomized, double-blind, placebo-controlled, phase II proof-of-concept clinical trial.

Key messages:

- In this small proof-of-concept randomized double-blind clinical trial, the PLP10 treatment statistically significantly reduced the ARR, and the risk of sustained disability progression without any reported serious adverse events.
- Overall, a total of 41 (51%) patients completed the 30-month trial. For the per-protocol analysis of ~~the two-year~~ primary end point, ~~8 relapses were recorded in the PLP10 group (n=10) (0.40 ARR) versus 25 relapses in the placebo group (n=12) (1.04 ARR), we observed~~ a 64% ~~adjusted~~ relative rate reduction for the PLP10 group (RRR 0.36, 95% CI 0.15 to 0.87, p=0.024). ~~In a subgroup analysis that excluded patients on monoclonal antibody (natalizumab) treatment, the observed adjusted RRR became stronger (72% over the two years (RRR 0.28, 95% CI 0.10 to 0.79, p=0.016). Per-protocol analysis for the secondary outcome at two years, time to disability progression, was significantly longer only with PLP10.~~ The cumulative probability of disability progression at two years was 10% in the PLP10 group and 58% in the placebo group (unadjusted log-rank p=0.019). ~~In a subgroup analysis that excluded patients on natalizumab the cumulative probability of progression was 10% for the 10 patients in the PLP10 group and 70% for~~

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the 12 patients in the placebo group, a relative 86% decrease in the risk of sustained progression of disability in the PLP10 group (unadjusted log-rank $p=0.006$; adjusted hazard ratio, 0.11; 95% CI 0.01 to 0.97, $p=0.047$).

PLP10's ingredients formulation (specific PUFA, MUFA, SFA, gamma-tocopherol, vitamin E and vitamin A), has probably the ability to interfere with the repertoire of mechanisms involved in MS pathogenesis as well as with the recovery mechanisms, neuroprotection and remyelination.

This proof of concept clinical study might be indicative of new treatment approach (holistic) of chronic complex multifactorial diseases and especially of MS prevention and treatment and of a new epoch of novel drug development.

Strengths and limitations of this study:

- The randomisation, blinding, the use of placebo, the definite inclusion/exclusion criteria and primary/secondary end points, along with the 30 month duration of the study, as well as the inclusion of a 6-month normalisation (chronotherapy) period allow for an appropriate overview of the safety and efficacy of the assessed interventions.
- The small sample size and the high rate of drop-outs (due to the palatability of the formula) are the limitations associated with the present study.

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Figure 1. Study Flowchart

Figure 2. Omega-6 and omega-3 PUFA, their respective metabolic derivatives and their possible effects on inflammation.

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After consumption, the PUFAs are metabolized via several pathways (not shown) to active compounds that mediate inflammation and products that promote resolution of inflammation.

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Abbreviations: PL, phospholipid; IFN- γ , interferon γ ; IL-2, interleukin 2; NF κ B, nuclear factor kappa B; PGE2, prostaglandin E2; PPAR γ , peroxisome proliferator-activated receptor γ ; PUFAs, polyunsaturated fatty acids; TGF β , transforming growth factor β ; TNF, tumor necrosis factor; COX, cyclooxygenase; hydroxyeicosatetraenoic acid; HPETE, hydroperoxyeicosatetraenoic acid; LOX, lipoxygenase; LT, leukotriene; PG, prostaglandin; TX, thromboxane; RXR- γ , retinoid X receptor-gamma; Nrf2, nuclear respiratory factor; MMP, metalloproteinase.

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Figure 3. Panel A demonstrates the ARR of all-time on-study patients during the 24mo pre-treatment (baseline ARR) and at different on-study intervals (6, 12, 18, 24 mo) per treatment arm. **

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7 1156 Panel B demonstrates the ARR of all-time on-study population between 0-6, 6-12, 6-18, and
8 1157 6-24 mo period intervals, of PLP10 vs. placebo group. **

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11 1158 Panel C demonstrates the ARR of all-time on-study population of PLP10 vs. placebo group at
12 1159 baseline, during 1st year, and during the 2-year on-treatment. **

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16 1160 Panel D demonstrates the dispersion of relapses throughout the 2-year period of all-time on-
17 1161 study (excluding patients on natalizumab) of PLP10 (n=10) vs. placebo (n=10). Placebo
18 1162 shows an irregular dispersion of relapses in relation to PLP10 group with linear increasing
19 1163 trend while PLP10 shows a stabilized linear trend. By using the per-protocol model where
20 1164 patients on natalizumab were excluded, we could compare the number of relapses on a same
21 1165 number of patients.

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28 1166 ** Including the patients on natalizumab.

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31 1167 **Figure 4.** Panel 4A demonstrates the Kaplan–Meier plot of the time to sustained progression
32 1168 of disability among all-time on-study patients, excluding patients on natalizumab, receiving
33 1169 intervention A, PLP10 and C as compared with placebo. PLP10 reduced the risk of sustained
34 1170 progression of disability by 86% over two years (p=0.006). Intervention formula A reduced
35 1171 the risk of sustained progression of disability by 53% (p=0.266) and intervention formula C
36 1172 by 67% (p=0.061).

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43 1173 Panel 4B demonstrates the Kaplan–Meier plot of the time to sustained progression of
44 1174 disability among ITT population receiving intervention A, PLP10 and C as compared with
45 1175 placebo. PLP10 reduced the risk of sustained progression of disability by 71% over two years
46 1176 (p=0.052, trend). Intervention formula A reduced the risk of sustained progression of
47 1177 disability by 22% (p=0.727) and intervention formula C by 40% (p=0.447).

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1178 **Figure 5.** Mean change in expanded disability status scale score as a function of visit

1179 number. Values are expressed as mean \pm standard error of the mean (s.e.m.)

1180 Including patients on natalizumab

1181 Excluding patients on natalizumab

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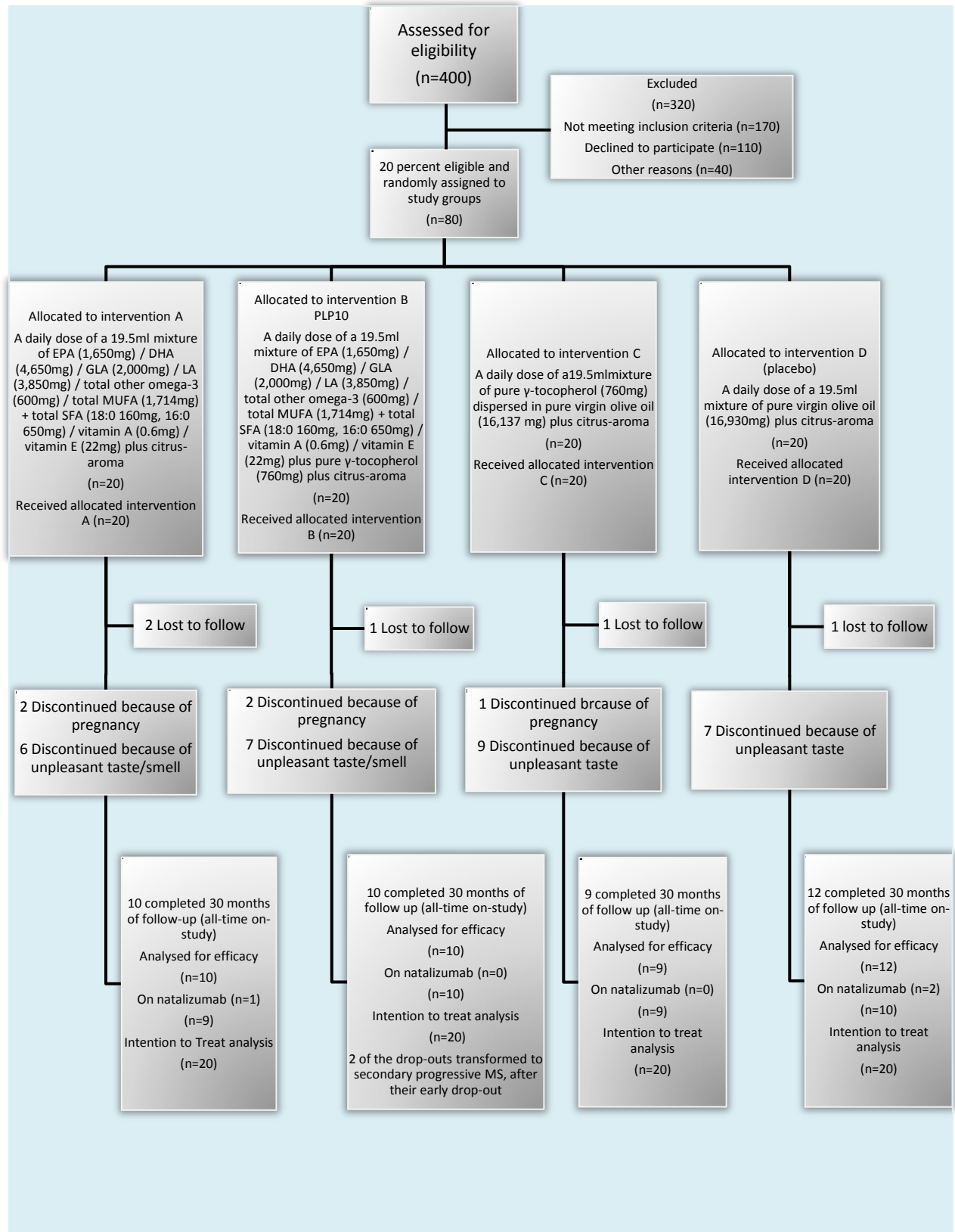
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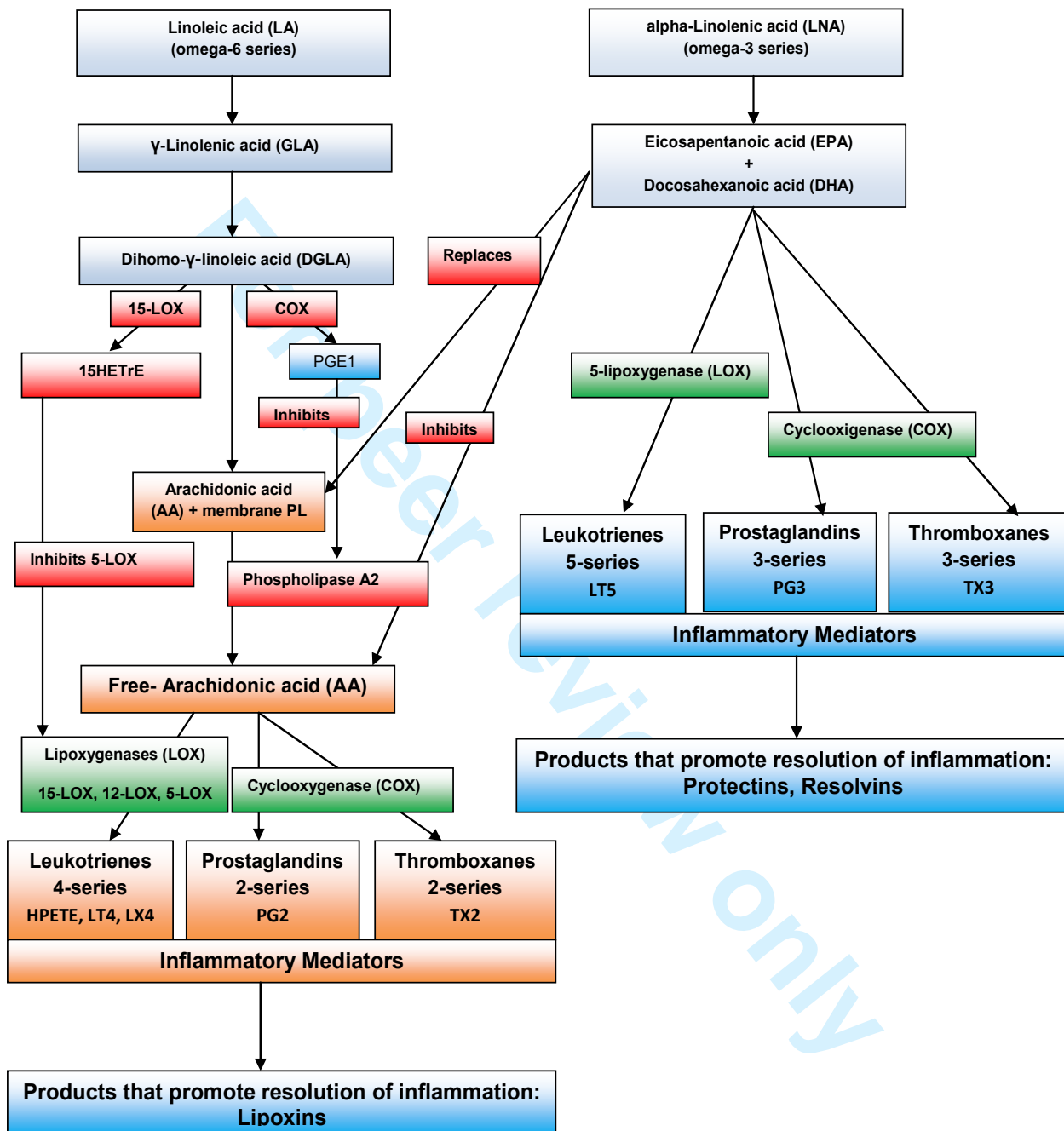
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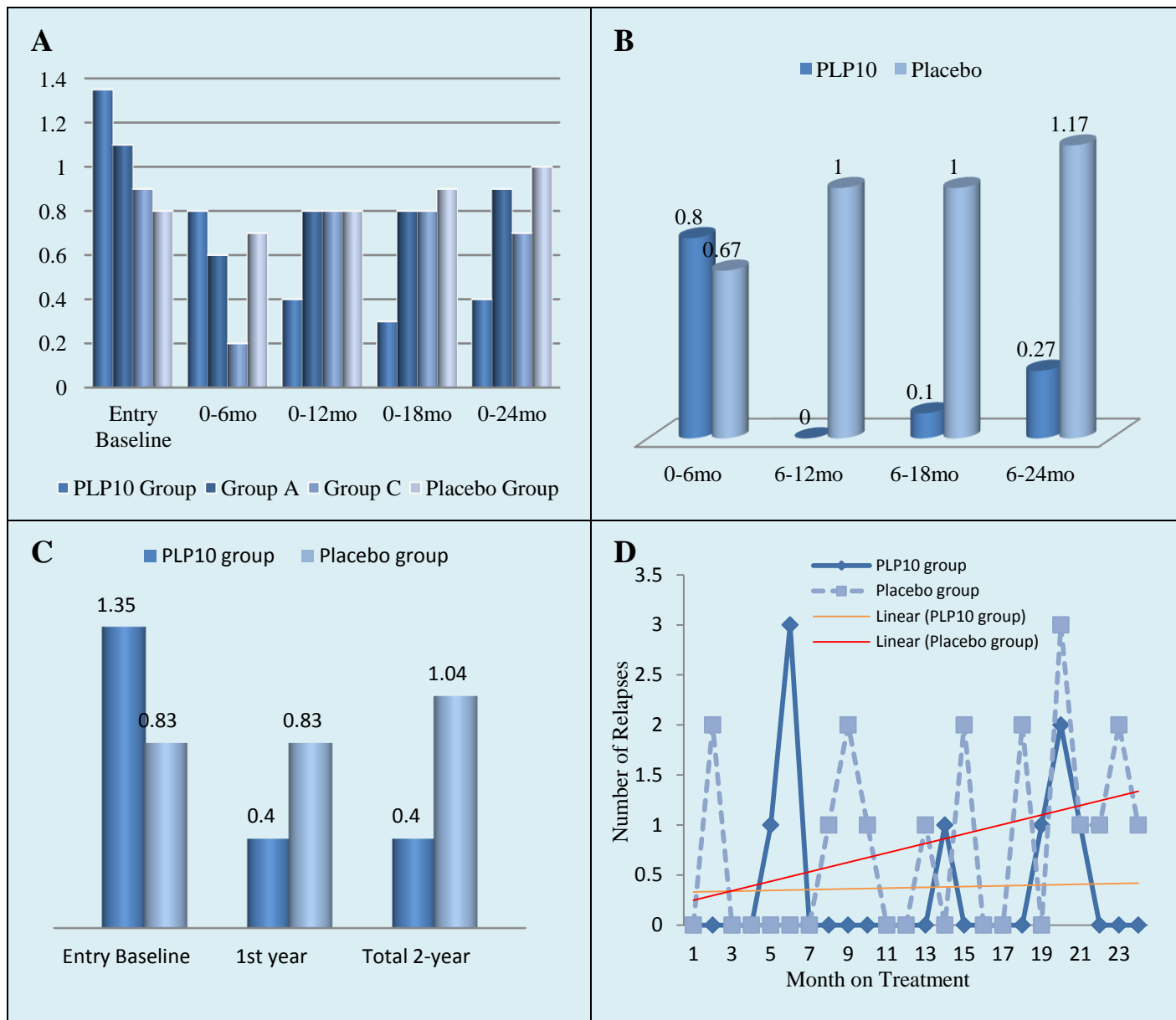


Omega-6 and omega-3 PUFA Consumption through Diet

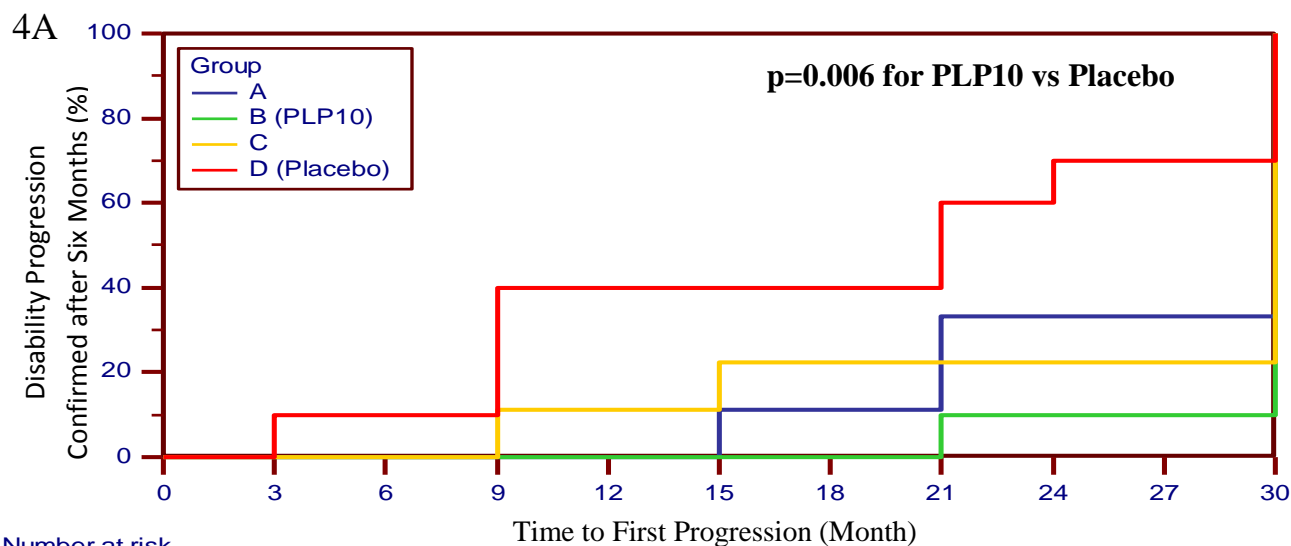


Possible effects on inflammation:
 Reduce IFN-γ production; Reduce IL-2 production; Increase TGFβ activity; Increase PGE2 activity; Inhibit arachidonic acid; Reduce TNF levels; RXR-γ and PPARγ agonist; NFκB expression; stimulate Nrf2; reduce MMP-2, -3, -9, and -13

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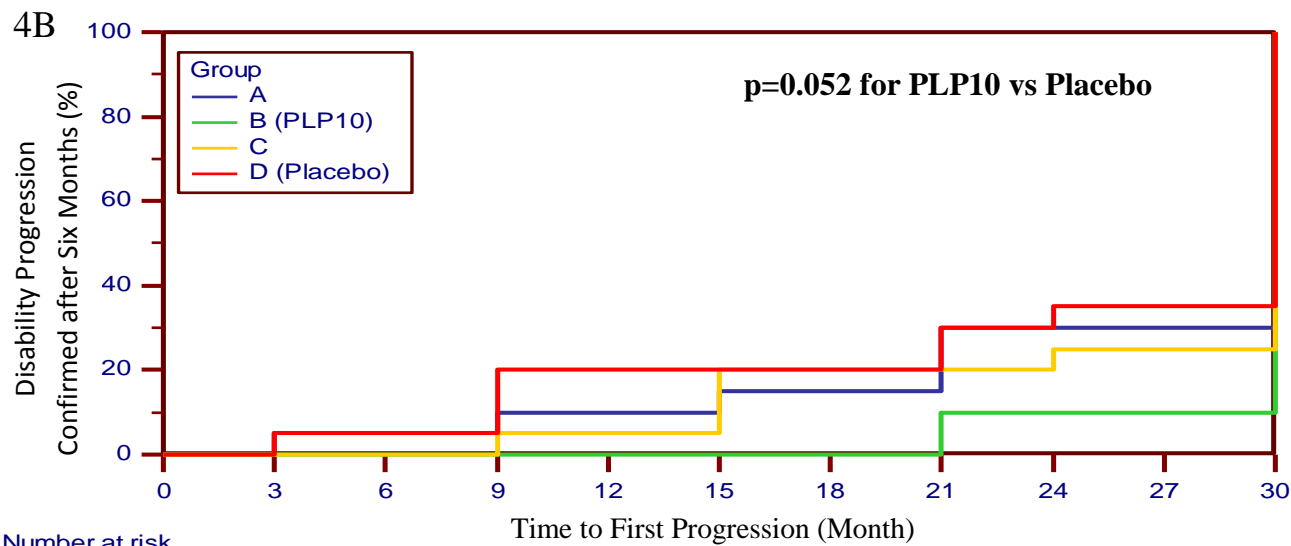


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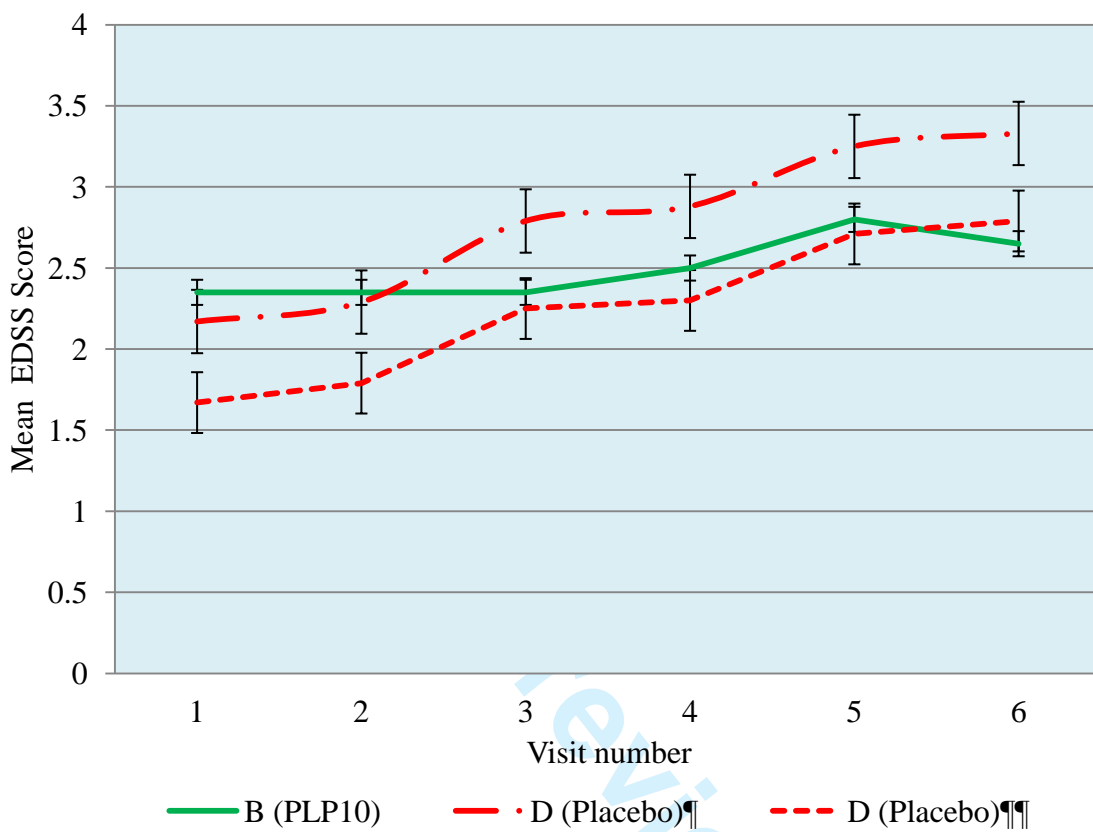
Number at risk

Group	0	3	6	9	12	15	18	21	24	27	30
Group: A	9	9	9	9	9	8	8	6	6	6	6
Group: B (PLP10)	10	10	10	10	10	10	10	9	9	9	9
Group: C	9	9	9	8	8	7	7	7	7	7	7
Group: D (Placebo)	10	9	9	6	6	6	6	4	3	3	3



Number at risk

Group	0	3	6	9	12	15	18	21	24	27	30
Group: A	20	19	19	18	18	17	17	14	14	14	14
Group: B (PLP10)	20	20	20	20	20	20	20	18	18	18	18
Group: C	20	20	20	19	19	16	16	16	15	15	15
Group: D (Placebo)	20	19	19	16	16	16	16	14	13	13	13



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3 **1 Supplementary Information**
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5 **2 Table of Content**
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34 **Supplementary Information Methods 1**

35 **Intervention and study rational.** We are using high dosage of 1:1 omega-3:omega-6
36 polyunsaturated (PUFA) aiming to overpass and normalize global diet regional traditions and
37 habits in relation to abnormal PUFA/saturated fatty acids (SFA)/monounsaturated fatty acids
38 (MUFA) daily ratio consumption irrespective of the quantities consumed; and enough to
39 equilibrate patients' diet in line with the recommended more physiologic omega-3:omega-6
40 ratio of about 1:1-4 wt/wt as reported by Simopoulos, 2002. In addition to correct existing
41 deficiencies, cell membrane abnormalities, specifically of the immunopathological system
42 and blood mononuclear peripheral cells, and high enough for availability and immediate
43 ongoing modulation of the involved pathogenic mechanisms and network of events in MS.
44 The high dosage is also required to overpass the quantity limitations, previously discussed, of
45 diet-consumed PUFAs for cellular incorporation, especially in the central nervous system
46 (CNS) of adults. Additionally, fatty acids (FAs) must first cross the intestinal epithelium
47 before reaching the different tissues, where digestion and absorption constitute further
48 problems in their availability (Carlier, H, 1991). Omega-3 PUFA are used in re-esterified
49 form to eliminate unwanted disturbances, at the sides of action, by other fatty acids and
50 molecules present in crude fish oils but also to increase the bioavailability of the FA since
51 triglycerides have been shown to be associated with much higher bioavailability (Dyerberg et
52 al, 2010). Linoleic (LA) and gamma linolenic acid (GLA) are essential structure molecules
53 and important for any physiological (re)generation of cell membrane. GLA quantity is
54 doubled to LA to ensure high direct production of dihomo-gamma-linolenic acid (DGLA),
55 from GLA when LA cannot be metabolized, due to desaturase deficiency or malfunction.
56 Such a reduced capacity to convert LA to GLA has been associated with aging, diabetes,
57 alcoholism, atopic dermatitis, premenstrual syndrome, rheumatoid arthritis, cancer and
58 cardiovascular diseases (Bolton-Smith et al, 1997; Horrobin, 1990; Leventhal et al, 1993).
59 This is going to result in the increase of DGLA relative to arachidonic acid (AA) with DGLA
60 promoting production of prostaglandin (PG)E1 but also inhibition of phospholipase (PL)A2:
61 two major reasons and rational for their use. If other metabolic problems are involved within
62 the omega-6 series and the normal metabolites are not produced then the eicosapentaenoic
63 acid EPA available in PLP10 will substitute the function of DGLA, as a competitive inhibitor
64 of AA for PLA2. In both cases the pro-inflammatory leucotrienes, prostaglandines of the 2-
65 series (PG2) and thromboxanes of the 2 series including the platelet-activator factor (PAF)
66 will be attenuated. The synthesis of AA from DGLA by $\Delta 5$ desaturase promoted by LA/GLA
67 supplementation is very limited in humans as a result of limited activity of the enzyme
68 (Yang-Yi & Robert, 1998). AA in the body is mostly available through diet. EPA and
69 docosahexaenoic acid (DHA) are both physiologically important and crucial structured
70 molecules able to substitute excess AA and SFA within the cell membranes. EPA will
71 contribute to the inhibition (competitive to AA) of PLA2, joining the co-supplied omega-6
72 PUFA but will also participate in the production of anti-inflammatory leukotrienes,
73 prostaglandins of the 3-series (PG3) and thromboxane (TX3) along with DHA, both found in
74 the PLP10 intervention. Moreover EPA will replace AA of the membrane phospholipids and
75 both omega-3 PUFA will contribute replacing abnormal quantities of SFA and excess AA.
76 DHA is used in 3:1 ratio to EPA to cover any possible inabilities of EPA to be metabolized,
77 high enough to strongly promote high production of the aforementioned anti-inflammatory

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3 78 eicosanoids and cytokines and to be incorporated into CNS cell membranes where DHA
4 79 should be the major PUFA present, replacing other FA, probably saturated and excess of AA.
5 80 EPA, DHA, LA and GLA along with the rest of the other ingredients used (“other” omega-3
6 81 PUFA, SFA and MUFA that are usually found in the cell membranes of healthy people in
7 82 limited quantities) in the intervention regimen are for their availability as minor structural
8 83 constituents of physiological cellular membranes integrity, fluidity and overall function as
9 84 building blocks for myelin repair and/or myelination. Furthermore the PUFA used within the
10 85 cocktail intervention aimed to manipulate all other pathophysiological pathways that are
11 86 reported to be able to: as previously discussed including gene transcription for
12 87 neuroprotection and remyelination. Furthermore, PUFA are used to ensure the integrity of
13 88 blood brain barrier (BBB) and to modulate the gelatinases responsible for the T cell migration
14 89 within the CNS. Three different antioxidant vitamins (vitamin E, mostly as alpha-tocopherol,
15 90 gamma (γ)-tocopherol (vitamin E isoform) and vitamin A) are used in the regimen
16 91 preparation to support the cellular antioxidant defenses but also to protect peroxidation of the
17 92 supplied increased amounts of PUFA. Alpha-tocopherol low-molecular-weight antioxidants
18 93 will contribute to radical scavenging, interfering with gene transcription, protein expression,
19 94 enzyme activity and metal chelation (van Meeteren et al, 2005). Vitamin E (alpha tocopherol)
20 95 and vitamin A are used as antioxidants for the protection of the excess supplemented PUFA,
21 96 with alpha-tocopherol been demonstrated to protect against peroxynitrite-induced oxidative
22 97 damage, as well as able to efficiently detoxify hydroxyl, perhydroxyl and superoxide free
23 98 radicals (the elevated reactive oxygen species (ROS)); each one with different mechanism of
24 99 action, increasing the effect capability (Vatassery et al, 1998b; van Meeteren, 2005). Gamma-
25 100 tocopherol is used in high dosage since its half life is very short compared to alpha-
26 101 tocopherol and has been demonstrated to specifically protect against nitro-radicals.
27 102 Tocopherols can also exert non-antioxidant properties, including modulation of cell signaling
28 103 and immune function, regulation of transcription, and induction of apoptosis as previously
29 104 discussed (van Meeteren et al, 2005).

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39 105 PLP10 is the first preparation ever developed for MS therapy that is composed by the use of
40 106 all different previously discussed PUFA, MUFA, SFA in a cocktail preparation mixed with
41 107 the specific aforementioned antioxidant vitamins that have never been all together used
42 108 before within a specific formulation. The ingredients ratio, quality, structural form and
43 109 mostly the high dosage has never been before tested. Furthermore, the knowledge and
44 110 chronotherapy as well as other unique limitations associated with the individual molecules
45 111 used, have never been accounted, discussed, proposed or reported for any previous
46 112 therapeutic regimen.

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50 113 Through systems medicine therapeutic philosophy, by the use of PLP10, potentially MS
51 114 patients have the opportunity to be treated holistically, by natural source isolated molecules,
52 115 demonstrated as able of affecting and modulating all known pathophysiological,
53 116 immunopathological, habitual, gene related factors; thus the dynamic interconnected complex
54 117 network of events simultaneously. Possibly synergistic effects between PLP10 ingredients are
55 118 also feasible. Moreover we can speculate that treatment efficacy of PLP10, when used as

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3 119 adjunct to existing pharmaceuticals produced by reductionism, can be proven therapeutically
4 120 superior to any available treatment for MS.
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160 **Supplementary Information Methods 2**

161 **Interventions specifications.** The specific omega-3 (re-esterified glycerides) and omega-6
162 (glycerides) raw materials were purchased according to the required interventions' PUFA-
163 fraction specification (molecular structure, quantity/ratio and quality) with vitamin E (alpha-
164 tocopherol) used as antioxidant stabilizer by the supplier. The vitamins and masking aroma
165 were purchased separately. The mixing of fractions to the final required intervention-
166 composition specification was always performed by the same team of scientists under the
167 supervision of the involved medical biochemist and lipidology specialist, under appropriate
168 conditions every six months. Interventions were stored refrigerated in dark until use.

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170 The ratios of the different ingredients used were as follows: omega-3, EPA to DHA (about 1
171 to 3 wt/wt), omega-6, LA to GLA (about 2 to 1 wt/wt), omega-3 (EPA + DHA) to omega-6
172 (LA + GLA) (about 1 to 1 wt/wt). The total omega-3 (EPA + DHA + "other" omega-3
173 PUFA) used as re-esterified triglycerol (minimum value 60%), diglyceride (about 33%),
174 monoglyceride (about 2%) structural form mixture and about 2% ethyl ester structural form,
175 with no less than 80% re-esterified triglycerol content to be DHA and EPA as a result of
176 PUFA triglycerides re-esterification of fish body oils. The "other" group of omega-3 on the
177 re-esterified glycerols included the 18:3 (alpha-linolenic acid), 18:4 (stearidonic acid), 20:4
178 (eicosatetraenoic acid) and 22:5 (docosapentaenoic acid) PUFA. The fraction of omega-6
179 (LA + GLA) used as triglycerides with no less than 50-65% triglycerol content to be LA and
180 GLA in a ratio of 2 to 1 with 18:1 (oleic acid) 14-20% and 20:1 (eicosenoic acid), 22:1
181 (docosenoic acid), 24:1 (tetracosenic acid) as additional monounsaturated fatty acids and
182 minor quantities of 16:0 (palmitic acid) 4-16%, 18:0 (stearic acid) 2-5% saturated fatty acids
183 from Borage oil source. The vitamins used were vitamin A as beta-carotene, vitamin E
184 (alpha-tocopherol) and pure gamma-tocopherol (vitamine E isoform). Citrus extract was used
185 as masking aroma and pure virgin olive oil as delivery vehicle.

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187 **The daily intervention formula agent dosages were:**

188 **Intervention formula A** daily dosage: EPA (1650mg) / DHA (4650mg) / GLA (2000mg) /
189 LA (3850mg) / total other omega-3 (600mg) / total monounsaturated fatty acids (MUFA)
190 (18:1 1300mg, 20:1 250mg, 22:1 82mg, 24:1 82mg) + total saturated fatty acids (SFA) (18:0
191 160mg, 16:0 650mg) / vitamin A (0.6mg) / vitamin E (22mg).

192 **Intervention formula B (PLP10)** daily dosage: EPA (1650mg) / DHA (4650mg) / GLA
193 (2000mg) / LA (3850mg) / total other omega-3 (600mg) / total MUFA (18:1 1300mg, 20:1
194 250mg, 22:1 82mg, 24:1 82mg) + total SFA (18:0 160mg, 16:0 650mg) / vitamin A (0.6mg) /
195 vitamin E (22mg) / gamma- tocopherol (γ -tocopherol) (760 mg).

196 **Intervention formula C** daily dosage: γ -tocopherol (760 mg) (in 16137 mg pure virgin olive
197 oil as a vehicle).

198 **Intervention formula D** daily dosage: pure virgin olive oil (16930mg).

199 Citrus aroma was added in each intervention formula to make up a total dosage of 19.5ml of
200 solution per day.

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3 201 The specific omega-3 related fraction, according to specifications required for the
4 202 interventions was prepared and purchased from EPAX AS, Aalesund, Norway; as re-
5 203 esterified glycerides from fish body oils as a source. The specific omega-6 PUFA, MUFA
6 204 and SFA related fraction, according to required specifications, was prepared and purchased
7 205 from Goerlich Pharma International GmbH, Edling, Germany, as triglycerides from Borage
8 206 seed oil (organic, cold pressed) "*Borago officinalis*" as a source. Both omega-3 and omega-6
9 207 fractions were delivered stabilized by the producer (vitamine E (alpha-tocopherol) ~ 4.5 mg/g
10 208 was used as antioxidant).

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14 209 Vitamins: vitamin A as beta-carotene (HealthAid Ltd., Middlesex, United Kingdom) and pure
15 210 gamma-tocopherol (Tama Biochemical Co. Ltd., Shinjuku-ku Tokyo, Japan).

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18 211 Citrus aroma (Givaudan Schwaiz AG, Dubendorf, Switzerland).

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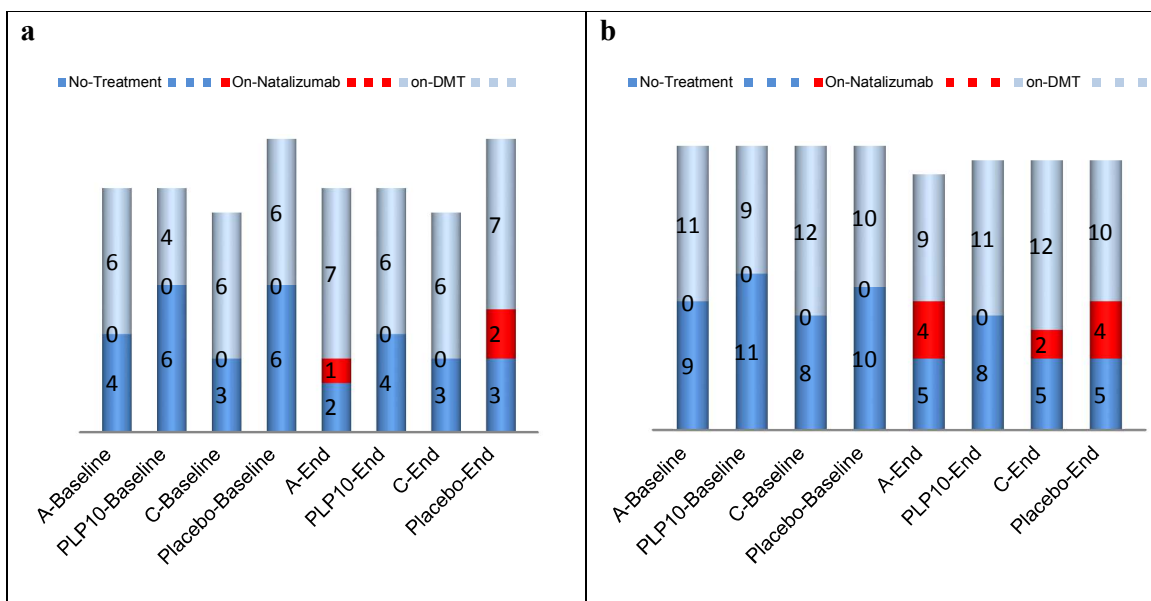
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Supplementary Information Figure 1 | Population on DMT and/or natalizumab. (a) Demonstrates the all-time on-study population per treatment arm that was receiving or not receiving DMT at entry baseline and the same population at the end of the trial (including patients on natalizumab). No statistical significant differences were calculated. The all-time on-study patients per treatment-arm that were receiving DMT at entry baseline were six patients out of ten (60%) within group A, four out of ten (40%) within PLP10 group, six out of nine (66%) within group C and six out of 12 (50%) within placebo. When the study completed, 80% of the patients in group A, 60% in PLP10 group, 66% in group C and 75% of the patients in placebo ended up on treatment. Within group A one out of eight and within placebo two out of nine patients on DMT transferred on natalizumab. (b) Demonstrates the total randomized population per treatment arm that was receiving or not receiving DMT at entry baseline and the same population at the end of the trial without lost to follow (including patients on natalizumab). A total of 61% of group A patients were on DMT at entry baseline and became 72% at the end; for PLP10 group 41% and became 53%; for group C 73% and became 74% and for placebo 53% and became 74% at study completion. At the end: for group A, four out of 13 patients on DMT transferred on natalizumab; for PLP10 group no patient was on natalizumab; for Group C two out of 14 patients on DMT transferred on natalizumab; and for placebo group four out of the 14 patients on DMT transferred on natalizumab. No significant differences measured at entry baseline between the groups.

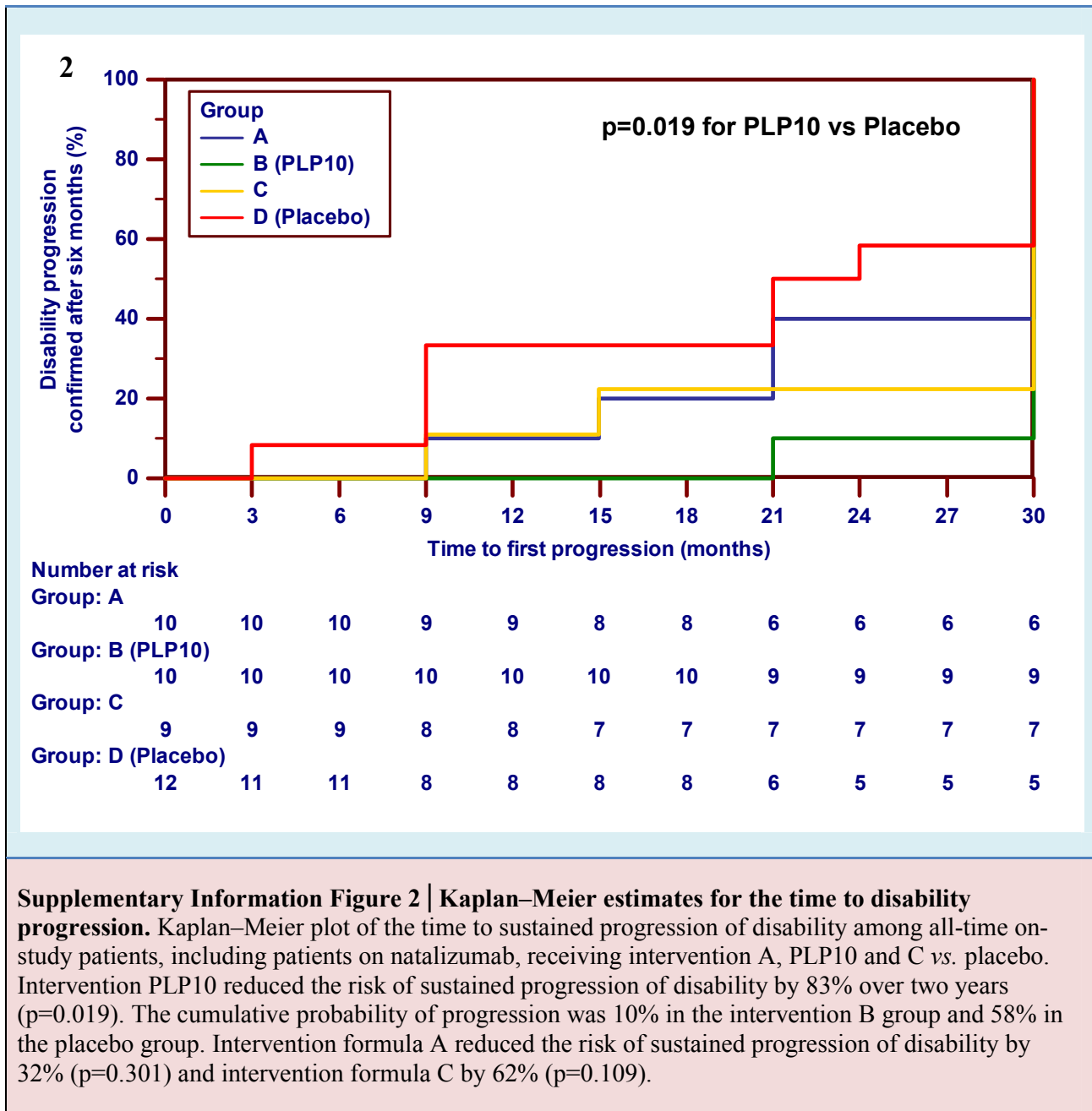
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Checklist of Items for Reporting Trials of Nonpharmacologic Treatments*

Section	Item	Standard CONSORT Description	Extension for Nonpharmacologic Trials	Reported on Page No.
Title and abstract†	1	How participants were allocated to interventions (e.g., “random allocation,” “randomized,” or “randomly assigned”)	In the abstract, description of the experimental treatment, comparator, care providers, centers, and blinding status	1,3,4
Introduction				
Background	2	Scientific background and explanation of rationale		5 to 8
Methods				
Participants†	3	Eligibility criteria for participants and the settings and locations where the data were collected	When applicable, eligibility criteria for centers and those performing the interventions	8, 13,15
Interventions†	4	Precise details of the interventions intended for each group and how and when they were actually administered	Precise details of both the experimental treatment and comparator	9,10,11, Table 1 p.28, Appendix p. 5,6
	4A		Description of the different components of the interventions and, when applicable, descriptions of the procedure for tailoring the interventions to individual participants	10, Table 1 p.28, Appendix p.5
	4B		Details of how the interventions were standardized	9,10, Table 1 p.28, Appendix p.5
	4C		Details of how adherence of care providers with the protocol was assessed or enhanced	9
Objectives	5	Specific objectives and hypotheses		7,8
Outcomes	6	Clearly defined primary and secondary outcome measures and, when applicable, any methods used to enhance the quality of measurements (e.g., multiple observations, training of assessors)		12
Sample size†	7	How sample size was determined and, when applicable, explanation of any interim analyses and stopping rules	When applicable, details of whether and how the clustering by care providers or centers was addressed	14

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5	Randomization– sequence generation†	8	Method used to generate the random allocation sequence, including details of any restriction (e.g., blocking, stratification)	When applicable, how care providers were allocated to each trial group
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8	Allocation concealment	9	Method used to implement the random allocation sequence (e.g., numbered containers or central telephone), clarifying whether the sequence was concealed until interventions were assigned	9
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12	Implementation	10	Who generated the allocation sequence, who enrolled participants, and who assigned participants to their groups	9
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15	Blinding (masking)†	11A	Whether or not participants, those administering the interventions, and those assessing the outcomes were blinded to group assignment	Whether or not those administering co- interventions were blinded to group assignment
16				9,10
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20		11B		If blinded, method of blinding and description of the similarity of interventions†
21				9,10,Appendix p.5
22	Statistical methods†	12	Statistical methods used to compare groups for primary outcome(s); methods for additional analyses, such as subgroup analyses and adjusted analyses	When applicable, details of whether and how the clustering by care providers or centers was addressed
23				13, 14, 15
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26	Results			
27	Participant flow†	13	Flow of participants through each stage (a diagram is strongly recommended)--- specifically, for each group, report the numbers of participants randomly assigned, receiving intended treatment, completing the study protocol, and analyzed for the primary outcome; describe deviations from study as planned, together with reasons	The number of care providers or centers performing the intervention in each group and the number of patients treated by each care provider or in each center
28				15 Fig 2
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35	Implementation of intervention†	New item		Details of the experimental treatment and comparator as they were implemented
36				10,15,16 Appendix p..5,
37	Recruitment	14	Dates defining the periods of recruitment and follow-up	11,15
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39	Baseline data†	15	Baseline demographic and clinical characteristics of each group	When applicable, a description of care providers (case volume, qualification, expertise, etc.) and centers (volume) in each group
40				16,Table 2
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Numbers analyzed	16	Number of participants (denominator) in each group included in each analysis and whether analysis was by “intention-to-treat”; state the results in absolute numbers when feasible (e.g., 10/20, not 50%)		15,16
Outcomes and estimation	17	For each primary and secondary outcome, a summary of results for each group and the estimated effect size and its precision (e.g., 95% confidence interval)		15 to 20
Ancillary analyses	18	Address multiplicity by reporting any other analyses performed, including subgroup analyses and adjusted analyses, indicating those prespecified and those exploratory		20
Adverse events	19	All important adverse events or side effects in each intervention group		20
Discussion				
Interpretation†	20	Interpretation of the results, taking into account study hypotheses, sources of potential bias or imprecision, and the dangers associated with multiplicity of analyses and outcomes	In addition, take into account the choice of the comparator, lack of or partial blinding, and unequal expertise of care providers or centers in each group	21
Generalizability†	21	Generalizability (external validity) of the trial findings	Generalizability (external validity) of the trial findings according to the intervention, comparators, patients, and care providers and centers involved in the trial	22
Overall evidence	22	General interpretation of the results in the context of current evidence		22 to 26

*Additions or modifications to the CONSORT checklist. CONSORT = Consolidated Standards of Reporting Trials.

†This item was modified in the 2007 revised version of the CONSORT checklist.