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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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Abstract

Objective To assess whether our three novel interventions, formulated based on systems 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.

Results The per-protocol, proof-of-concept, analysis demonstrated a 64% adjusted relative reduction in ARR at two years for PLP10 versus placebo (P=0.024). Regarding the secondary endpoint, a relative reduction of 86% in the risk of sustained progression of disability was observed within the PLP10 group (p=0.047). No adverse events were reported. Interventions A and C showed no significant efficacy.

Conclusions PLP10 treatment significantly reduced the ARR, and the risk of sustained disability progression without any adverse or significant side effects.

Trial registration International Standard Randomized Controlled Trial, number ISRCTN87818535.

Introduction

Multiple sclerosis (MS) is a complex multifactorial disease that results from interplay between as yet unidentified environmental factors and susceptibility genes.[1-3] Together, these factors trigger a cascade of events, involving engagement of the immune system, inflammatory injury of myelin, axons and glia, functional recovery and structural repair, gliosis, and neurodegeneration.[4] The bio-mechanisms involved are: immune-mediated inflammation, oxidative stress and excitotoxicity.[5-9] These mechanisms may all contribute to oligodendrocyte and neuronal damage and even cell death, hence promoting disease progression. The increasing prevalence of 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.

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

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

cellular antioxidant defense mechanisms have been reported for MS patients.[11] The cause of these PUFA deficiencies is not entirely clear and may involve metabolic and nutritional alterations.[11]

Increased or uncontrolled inflammation contributes to several different acute and chronic diseases and it is characterized by the production of inflammatory cytokines, arachidonic acid (AA)–derived eicosanoids (prostaglandins (PGs), thromboxanes (TXs), leukotrienes (LTs), and other oxidized derivatives), other inflammatory agents such as reactive oxygen species (ROS) , nitric oxide (NO), and adhesion molecules (Fig 1).[12] During inflammation glutamate homeostasis is altered by activated immune cells releasing increased quantities of glutamate that can result in over activation of glutamate receptors and in return excitotoxic oligodendroglial death.[7, 13] As such, among others, membrane-related pathology, immune-mediated inflammation, oxidative stress and excitotoxicity provide potentially useful combined targets for intervention in MS.

In vitro and in vivo studies have demonstrated that dietary EPA, DHA, LA, and GLA can be implicated and modulate almost all known complex network of events and pathways repertoire in MS pathophysiology. Brain membrane fatty acid composition can be modified with dietary supplementation, but the process has been showed to be age dependent (it takes much longer in adults vs. developing brains) as well as possibly dependent on the quantities of the dietary/supplemented PUFAs.[14] Both human and animal studies proved that diets high in DHA and EPA increase the proportion of these PUFA in the membranes of inflammatory cells and reduce the levels of AA.[12, 15] The anti-inflammatory properties of omega-3 include production of PGs and TXs of the 3-series and LTs of the 5-series (Fig 1).[14, 16] Resolvins and protectins are biosynthesized from omega-3 fatty acids via cyclooxygenase-2/lipoxygenase

(COX-2/LOX) pathways and they promote control of inflammation in neural tissues (Fig 1).[17-21] T-cell proliferation in acute and chronic inflammation can be reduced by supplementation with either omega-6 or omega-3 PUFA.[22] Furthermore, vitamin E is an important antioxidant that can interrupt the propagation of free radical chain reactions.[23] Vitamin E (alpha-tocopherol, an isoform of vitamin E) efficiently detoxifies hydroxyl, perhydroxyl and superoxide free radicals.[24] However γ-tocopherol (another isoform of vitamin E) seems to be more efficiently implicated in trapping NO radicals.[25] In addition alpha–tocopherol exerts non-antioxidant properties, including modulation of cell signaling and immune function, regulation of transcription, and induction of apoptosis.[26]

Moreover, omega-3 fatty acids electrophilic derivatives formed by COX-2, in activated macrophages can stimulate the nuclear respiratory factor (Nrf2) inducing the transcription of neuroprotective and antioxidant related genes, and can activate the peroxisome proliferator-activated receptor (PPAR)γ for anti-inflammatory response.[27-29] In animal studies, EPA and DHA proved to be endogenous ligands of RXRs, with positive effect on neurogenesis.[30] Additionaly, in 2008 Salvati and coworkers reported evidense of accelerated myelination in DHA- and EPA-treated animals.[32] Moreover, DHA and EPA are reported to significantly decrease the levels of metalloproteinases (MMP) -2, -3, -9, and -13 with a significant role in the migration of lymphocytes into the CNS by inducing disruption of the blood brain barrier (BBB), an important step in the formation of MS lesions.[33-39]

Based on the above, specific PUFA and antioxidant vitamins fulfill the criterion of biologic plausibility and have the potential to diminish MS symptoms severity and activity, even promoting recovery (remyelination).[11] Overall, PLP10 includes multiple ingredients

interacting with key interconnected components within functional network modules, each contributing a fraction of the effects of perturbations that cause the disease.[40]

In our phase II, single-center, randomized, double-blind, placebo-controlled, proof-of-concept clinical trial we intended to evaluate the therapeutic ability of PLP10 and of two other interventions (A and C) consisting of PLP10 constituent partial fractions versus placebo, when used on RRMS patients.

Methods

Patients

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

The study was conducted in accordance with the standards of the International Conference of Harmonization Guidelines for Good Clinical Practice. The protocol was developed by the investigators and it was approved by the Cyprus National Bioethics Committee and was overseen by an independent safety-monitoring committee evaluating the safety and over-all benefit-risk profiles. The adherence of care providers with the protocol was assessed by an external committee assigned by the funder of the project through reviews of case report forms. All patients gave written informed consent at the time of enrolment.

Randomisation and masking

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

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

other investigators, pharmacist, neuroradiologist and patients were masked to treatment allocation.

Procedures and end points

The specific omega-3 (re-esterified glycerides) and omega-6 (glycerides) raw materials were purchased according to the required interventions' PUFA-fraction specification (molecular structure, quantity/ratio and quality) with vitamin E (alpha-tocopherol) used as antioxidant stabilizer by the supplier. The vitamins and masking aroma were purchased separately. The mixing of fractions to the final required intervention-composition specification was always performed by the same team of scientists under the supervision of the involved medical biochemist and lipidology specialist, under appropriate conditions every six months. Interventions were stored refrigerated in dark until use. See Supplementary Information Methods 1 and 2 for intervention specification detailed description and study/intervention rational.

Participants were randomly assigned to receive a daily dose of a mixture of EPA (1,650mg) / DHA (4,650mg) / GLA (2,000mg) / LA (3,850mg) / total other omega-3 (600mg) / total MUFA (1,700mg) + total SFA (18:0 160mg, 16:0 650mg) / vitamin A (0.6mg) / vitamin E (22mg) (intervention A, group A); or composed mixture of pure γ-tocopherol (760mg) dispersed in pure virgin olive oil (16,137 mg) as delivery vehicle (intervention C, group C); or a mixture of intervention formula A with intervention C without the pure virgin olive oil (intervention B, named PLP10, group B); or placebo composed of pure virgin olive oil (16,930mg) (intervention D, group D). 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. The institution's pharmacist was responsible for the appropriate storage and handling

of the interventions to the individual participants. The interventions were taken orally once daily 30 minutes before dinner by a dosage calibrated cup for 30 months. The ingredients, ratio and dose have been selected based on their biophysical interrelation to the total known multiple MS causing factors, their biochemical importance and the role expected to play in the normalisation and treatment of the involved complex network of events in the disease pathophysiology. Moreover, the high intake dosage was used to overcome any abnormal dietary accumulation of related agents as a result of patients' food intake habits, irrespective of geographical origin, in relation to the daily consumption ratio of the total fatty acid intake; in order to end-up with omega-3 to omega-6 PUFA indicated physiological body ratio composition of 1:1 wt/wt.

The period beginning from July 1st 2007 (enrolment) until December 31st 2007 (entry baseline) was used for normalization period. This six-month normalization period would allow the interventions' agents to exert their beneficial effect (for the incorporation/normalization of cell membranes by oral PUFA, since they need four to six months to exert pivotal action on immune and neural cells, correction of antioxidant deficiency and body PUFA, and optimal normalization of EPA and DHA levels/ratio).[41-43] The study was completed on December 31st 2009 and the recording of relapses continued until December 31st 2010.

Depending on their clinical status and in accordance with the ethical issues governing clinical trials participants continued receiving the indicative regular available treatments, according to international guidelines with persistent evaluation of any side-effects and adverse events. The study was designed to end 30 months after enrolment and clinical assessments were scheduled at entry baseline, 3, 9, 15, 21 and 24 months on-treatment. Patients were also

clinically examined by the treating neurologist within 48 hours after the onset of new or recurrent neurologic symptoms.

The primary end point was the ARR at two years. A relapse was defined as new or recurrent neurologic symptoms not associated with fever or infection that lasted for at least 24 hours and accompanied by new neurologic signs. Relapses were treated with methyl-prednisolone at a dose of 1g intravenous per day, for three days followed by prednisone orally at a dose of 1mg/kg of weight per day on a tapering scheme for three weeks. The secondary end point at two years was the time to confirmed disability progression, defined as an increase of 1.0 or more on EDSS, confirmed after six months (progression could not be confirmed during a relapse). The final EDSS score was confirmed six months after the end of the study. A post-hoc analysis was performed assessing the proportion of patients free from new or enlarging T2 lesions on brain MRI scans at the end of the study for the per-protocol participants of the group receiving the highest effective intervention versus placebo. Comparison was made only versus the available archival MRI scans up to three months before the enrolment date. MRI scans were performed and blindly analyzed at an MRI evaluation centre. The patients continued to be followed for additional 12 months after completion of the trial and relapses were recorded. Finally, patients were strongly encouraged to remain in the study for follow-up assessments even if they had discontinued the study drug.

Blood samples were collected from all randomized patients at the time of enrolment, at every scheduled clinical assessment and during relapses. To check individual compliance with intake, the fatty acids composition of patients' red blood cells' membranes was determined, by gas chromatography, according to a standard protocol. The fatty acid analyses were performed after study termination and thus did not influence the blinding.

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

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

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

Statistical analysis

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

patients eligible for treatment in the trial. Sample size was strictly based on this subjects' availability parameter and the novelty of the assessed intervention.

Baseline characteristics were compared across all intervention groups by ANOVA or Kruskal-Wallis rank test for continuous variables and by an exact chi-squared test for categorical variables, as appropriate.

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

For the secondary end-point outcome, the time to disability progression, Kaplan–Meier curves were constructed. Progression to disability and time thereof was compared in a pair-wise fashion for the active interventions versus placebo by the log-rank test in the main analysis and by Cox proportional-hazards models with adjustment for baseline EDSS score, age and DMT in the supportive analysis. Each test was performed with a significance level of 0.05. Multivariate models considered all variables with $P < 0.1$ on univariate models. There was no overt violation of the proportionality assumption.

Both, per-protocol and intention to treat (ITT) analyses were performed, for different sets of research questions to be answered, and both are reported. Missing data of the five lost to follow patients were imputed by use of the last-observation-carried-forward (LOCF)

approach. Due to the proof-of-concept design of the study, the considerable non-adherence rate (49%) and the interpretation issues caused thereof regarding the ITT analysis, the per-protocol analysis considered being more informative and appropriate method approach to answer the research addressed questions of efficacy of the interventions when subjects were continuously following the protocol. All analyses were performed with STATA SE 10.0 (College Station, TX, USA). P-values are two-tailed.

Role of the funding source

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

Results

Study population

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

Among the 80 patients, 20 patients were randomly assigned to each of the three groups to receive the interventions and 20 to receive placebo (Fig 2). Baseline characteristics of both the ITT and the per-protocol populations were similar across groups (Table 1A and 1B). Total drop-out patients completed follow-up until study completion and were included in the ITT analyses (Table 3). Five patients were totally lost to follow before their first scheduled

visit and two patients dropped-out before their first scheduled visit progressed to secondary progressive MS. Fifteen patients dropped-out without successfully completing the "normalization" period including five pregnancies. Another 17 patients dropped-out early after entry baseline. Seven patients that dropped out were given monoclonal antibody treatment (natalizumab). Overall, a total of 41 (51%) patients completed the 42-month study (July 2007 through December 31st 2010, including the 12-month extended period) where one patient from group A and two from the placebo group transferred on natalizumab, and 39 (49%) patients either withdrew (drop-out) or lost to follow. Reasons for study-interventions discontinuation are listed in Figure 2.

Efficacy

Relapses

As a proof-of-concept trial we primarily needed to answer whether the interventions were effective for those MS patients who adhere to the assigned treatment, the per-protocol analysis.[45] For the sake of methodological comprehensiveness we also present the ITT analysis as a secondary analysis, to answer a different question, complementary to our core hypothesis; like what happened to MS patients who were placed on the interventions (the effect of assignment).[45] Otherwise, as a result of a high drop-out rate, an ITT analysis will not likely be able to show the superiority of an intervention even if it is effective.[45] In any instance, the proper approach of evaluating a study data is to understand what question prompted the research and assure that the analysis is appropriate for providing the answer whatever it is called. Both analyses can be performed for a study, using the results from the different analyses to answer different research questions.[45] These interventions are original, composed by a different treatment rational, the SM, never tested before and the important main concern was to evaluate their efficacy and safety based on the per-protocol treated MS

patients, without any peripheral noise. The question that had to be answered was: "what happens to the patients that are placed and stick on the specific treatment".

Regarding the per-protocol analysis, during the first year of treatment, the ARR was 0.80, 0.40, 0.78 and 0.83 for the four intervention groups respectively. During the second year, the ARR was 0.90, 0.40, 0.67 and 1.25 for the four intervention groups respectively. Overall, for the two-year primary end point, 8 relapses were recorded for the 10 patients in PLP10 group (0.40 ARR) versus 25 relapses for the 12 patients in placebo (1.04 ARR), a 64% adjusted relative rate reduction (RRR) for the PLP10 group (RRR 0.36, 95% confidence interval (CI) 0.15 to 0.87, p=0.024) (Tables 2A, 4 and Fig 3A and 3C). Excluding 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, Tables 2B and 4). Pair-wise comparisons for the other two groups against placebo did not yield statistically significant results (Tables 2A, 2B). The proportion of patients with ≤1 relapse for the two years on-study was higher in the PLP10 group than in the placebo group (90% *vs.* 42%, p=0.030, Table 4). Seeking to investigate further the observed difference, we compared the relapse rate during the 24 months before entry to the study to the 24 months on-treatment for each intervention group. We observed a statistically significant relative reduction in the ARR (70%) only in the PLP10 group (RRR 0.30; 95% CI 0.14 to 0.65, p=0.003, Table 2A); within-group comparisons for the three other groups ARR reduction was not significant and remained not significant when natalizumab treated patients were further excluded from the analysis. The effect of PLP10 through time at different time-windows versus placebo for all-time on-study patients is shown in Figures 3A to 3D. The ARR analysis, within time-windows, was not an assigned endpoint, but it could help in the process of evaluating parallel information as the time needed for a specific treatment intervention activity to be evident, as well as the efficacy

profile through time. PLP10 reached maximum effect within a year on-treatment (counting from the entry baseline) and remained stable at an ARR of 0.4, displaying a steadily reduced ARR with long free-relapse time-windows. These group B characteristics are considered important parameters of a successful MS treatment where the rule than the exception is the heterogeneity among patients' disease evolution. Specifically, Figure 3D demonstrates the dispersion of relapses throughout the 2-year period of all-time on-study (excluding patients on natalizumab) of PLP10 (n=10) *vs.* placebo (n=10). Placebo, in line with the existing knowledge of how relapse history works in relation to future relapses on MS patients (contagion phenomenon) indicates the expected linearly increased trend of the relapse incidence.[46] The same phenomenon was true for the groups A and C. Finally, during the 12 month post-study extended period (January 1st 2010 to December 31st 2010) all-time on-study patients that received PLP10, showed persistent benefit in the ARR compared to placebo (six relapses for the 10 subjects within PLP10, 0.6 ARR *vs.* 19 for the 12 subjects within placebo group, 1.58 ARR) indicating a statistically significant 62% adjusted relative rate reduction in the ARR for the PLP10 group (RRR 0.38, 95% CI 0.12 to 0.99, p=0.046).

Regarding the ITT analysis, within PLP10 group, none of the nine drop-out patients changed to natalizumab and two out of nine discontinued because of pregnancy, whereas two out of seven drop-out patients from the placebo group changed to natalizumab (a total of four patients within the placebo arm population were on natalizumab, including the two patients that transferred while all-time on-study versus none within PLP10 group (Supplementary 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 3A. 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 3B). 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 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 patients on natalizumab, there was an increased statistically significant difference between the PLP10 and the placebo group for the same analysis (p=0.006) (Fig 4A). At two years, the cumulative probability of progression was 10% in the PLP10 group and 70% in the placebo group, which represents a decrease of 60 percentage points or a relative 86% decrease in the risk of sustained progression of disability within 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 difference was observed for any comparison of the other two groups compared to placebo (Fig 4A and Supplementary Information Fig 2).

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

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

MRI

Over two years, the MRI results support the overall conclusion from the study that PLP10 has a positive effect on disease activity since only 29% from the PLP10 group as opposed to 67% from the placebo group developed new or enlarging T2 lesions (57% relative risk reduction). Excluding patients on natalizumab there is an increased relative risk reduction (64%) between PLP10 as opposed to placebo with 29% of patients on PLP10 and 80% on placebo with development of new or enlarging T2 lesions (Table 4).

Safety

Over the course of the 30 month study no significant adverse events were reported from any group. According to a questioner procedure the only aetiology for drop-outs was the palatability and smell of the formula preparations. Nausea was reported by two patients. No abnormal values observed on any of the biochemical and haematological blood tests. No allergic reactions reported.

Discussion

In this proof-of-concept clinical trial assessing the safety and efficacy of three cocktail intervention formulas in RRMS, we observed a significant benefit for the novel PLP10

intervention compared to placebo for both the ARR and the progression to disability. Our results include analyses pertaining to a total of 42 months study collected data, including the 12-month, free of intervention treatment, extension period. We focused on the per-protocol data analysis since it is the appropriate method to best provide the answer to the proof-of-concept trial-addressed question. The high drop-out rate was solely the result of formulas palatability, a common phenomenon in trials using oily interventions. We thus present our main per-protocol analysis, as well as a subgroup analysis excluding patients on natalizumab. We have found a statistically significant reduction in the ARR and the disability progression comparing not only patients on PLP10 versus placebo but also comparing the ARR of the PLP10 patients in the 24-month period prior to the study to the ARR of the 24 months on-study; the observed differences became larger when patients that received natalizumab (the most potent disease modifier) were excluded. The ARR decreased within a year on PLP10 and significantly remained stable until study completion. Statistically significant difference of ARR between patients on PLP10 versus placebo continued for the additional 12 month extended period (persistent effect) without significant difference on DMT. These clinical findings are supported by the results regarding the MRI analysis where the proportion of patients free from new or enlarging brain T2 lesions was also higher in PLP10 group versus placebo. The persistent effect within the extended period it is considered of major importance and supportive of the reults since it is in agreement with the very long washouts, reported necessary, for omega-3 fatty acids and especially DHA to return towards pretreatment values within the fatty acids of plasma, platelets, monocytes and red blood cells.[42] This study also provides important 30-month, placebo-controlled information about the safety of PLP10, A and C interventions, where no any severe or significant side-effects have been reported.

As medications used to treat MS become increasingly highly specific and potent, attention to safety is paramount. Current available treatments are products of reductionism, partially effective, associated with severe side effects without (re)myelinating or neuroprotective abilities. Interferons and glatiramer acetate, the most widely used first-line MS drugs available today, are associated with the least severe side-effects among MS therapies but they are reported with only 29-33% ARR reduction and with no significant effects on the progression of disability. Natalizumab as previously discussed and Fingolimod with 54% ARR reduction (without significant benefit on the progression of disability) are second-line drugs associated with severe side-effects.[47, 48]

No existing MS treatment has ever been designed as a result of SM concept approach or with a potential to effectively stimulate intrinsic remyelinating and neuroprotecting mechanisms or exert such an action. Now we propose that a holistic SM model approach has to be applied by synchronized action on all involved perturbed mechanisms. PLP10 has innovative characteristics like no any other intervention or medication tried before for MS treatment, with unique efficacy abilities through different mechanisms of action, probably by the synergistic effect of its constituent ingredients. PLP10 has all the characteristics of a medical food with the action to feed a normal metabolic process by supplying nutritional structural membrane precursors, building blocks, and vitamins from dietary sources that enhance remyelination and neuroprotection and simultaneously promote normalization of all cellular membranes lipid content. The intention is to normalize the specific nutritional requirements of the MS patients.

Different factors and molecular entities appear to be part of the possible aetiology for MS with specific PUFA and antioxidants found to be key substances related to all known

pathogenic and recovery mechanisms. But, it is well established that MS patients are characterized with significant deficiencies in antioxidant efficiency as well as in PUFA levels in blood and cellular membranes.[11, 49-51]

According to one hypothesis, the change in the ratio of omega-3 to omega-6, due to increasing consumption of omega-6 PUFA, meaning high accumulation of AA, in the Western diet, may be one of the major factors responsible for the increasing incidence of inflammatory diseases relative to populations. In the Western diet, the ratio of omega-3 to omega-6 is about 1:20–30; in populations that consume fish-based diets, the ratio is about 1:1–2.[52, 53] The intervention daily dose was aiming and believed to be high enough to restore/amplify body efficient antioxidant activity and ensure cellular membranes lipid profile normalization (PUFA content) and simultaneously potentiate involvement of the ingredients in the anti-inflammatory and recovery mechanisms. Diet fatty acid molecules need about six months period to exert their beneficial effect and this essential parameter was for the first time under consideration in our study design.[42] This chronotherapy parameter it is of major importance in line with the SM treatment philosophy and if it is not included in the trial design the possibility of misleading result evaluation greatly increases. In fact, considering that omega-3 supplementation can release and replace excess AA within the cellular membranes, we can speculate that an increased inflammatory activity can possibly result during the first six months of supplementation (during normalization period).

The maintenance of myelin requires continued turnover of its components throughout life.[54,55] In addition to the EPA, DHA, LA, and GLA, PLP10 was containing limited quantities of other structural PUFA, specific MUFA (mostly oleic acid) and SFA (palmitic and stearic acid), specifically to provide a direct source for neuronal cell membranes rehabilitation and

for (re)myelination and neuroprotection since they are all major components, precursors and building blocks of any new physiological myelin and cellular membranes in general. Assembly of the correct molecules into myelin membrane may be especially critical during active synthesis. Possibly, if critical constituents aren't available or are metabolically blocked, amyelination, dysmyelination or demyelination may ensue.[56]

The well known and established safety of the ingredients used and the protocol guidelines were supportive reasons for us to proceed with the clinical study even though with limitation on the pre-estimation of required trial sample size as it was discussed in method section. The adherence of the subjects is another issue but the duration of the study (42 months) is adding power to the results;[44] having the research questions been consciously and carefully approached and answered. Furthermore, the statistical methodologies used along with the appropriate adjustments, broadly accepted for MS clinical trials, power the findings, results, and significance. The baseline characteristics of the treatment arms could possibly be considered indicative of four very active groups of patients but that was the result of the limited number of RRMS population eligible for the study within Cyprus. On the other hand the balanced baseline characteristics without statistical differences, the statistical adjustments (for all important baseline parameters i.e. relapses, EDSS, age and DMT) and the randomization within four different groups are the safety valves against data misinterpretation. It is possible to question why DMTs efficacy cannot be emerged out of the data analysis, of the four treatment arms, and in accordance to their published values. We believe that the limited efficacy of the DMTs, the sample size and the statistical adjustments were strong limiting determining factors for such an indication to be countable. An additional argument is that the efficacy reported for the analysis of pre-treatment (24 months before entry baseline) vs on-trial ARR could be considered as potentially biased due to differences

of how relapses were defined during the course of a study compared to pre-treatment period; or due to regression to the mean or placebo effect. This analysis was performed as an additional exploratory analysis that we were able to do due to the availability of data. The relapses of the two pre-treatment years were drawn out of the patients' archival records by the same treating neurology involved in the study (MP), and according to the patients' hospitalization date for receiving intravenous methyl-prednisolone. This analysis was not used as a primary or a secondary end-point under investigation although it is usually reported by many clinical studies. As a matter of fact many early phase trials are based only on such an analysis (before vs after treatment results). In almost all MS trials the number of relapses within the two years before baseline is a factor under adjustment for the statistical analyses.[48] The inclusion of the post-hoc MRI analysis is another limiting factor that needs attention since it was used as an additional aside exploratory approach (due to study budget limitations it was not possible to be used as a formal endpoint); but the MRI evaluation was blinded and can be considered as representative of the randomized subjects within the treatment arms. As far as the regression to the mean and the placebo effect concerns we believe that the 6-month normalization period is an accountable and valuable eliminating factor of the possible effect; as well as the presence of four groups, where only the PLP10 treatment arm is associated with statistically significant efficacy vs placebo. It is a placebo-controlled study after all.

Our observations are consistent with the idea that simultaneous availability of specific PUFA along with other major membrane and myelin building blocks in combination with specific antioxidants, within optimum quantity, quality, ratio and structural form can possibly result to a more appropriate holistic therapy reducing MS disease activity. This is probably succeeded through synergistic and/or simultaneous effect on the interactions and dynamics of the most probable environmental and biological disease causing factors that induce complex biological

network of events for disease pathogenesis and evolution; as well as on the protective and reparative mechanisms. We can additionally speculate that the nature of the intervention formula cannot be prohibitive for its use as preventive regimen and does not preclude probable positive efficacy on the other types of MS, but has to be further investigated. A larger size multicenter clinical trial will better establish PLP10 place in the armamentarium of treatments for MS.

It is commonly accepted that nutrition is one of the possible environmental factors involved in the pathogenesis of MS, but its role as complementary MS treatment is unclear and largely disregarded.[57] Dietary antioxidants and fatty acids may influence the disease process in MS by reducing immune-mediated inflammation, oxidative stress and excitotoxic damage.[11] Present data reveal that healthy dietary molecules have a pleiotropic role and are able to change cell metabolism from anabolism to catabolism and down-regulate inflammation by interacting with enzymes, nuclear receptors and transcriptional factors.[57] The present study, for the first time provides strong link evidence between dietary, metabolic, immunological, and neurobiological aspects of MS after three quarters of a century of unsuccessful scientific efforts. This might probably be the beginning of opening new horizons and new avenues in the approach of MS prevention and treatment, and possibly of other multifactorial chronic diseases, including neurodegenerative and autoimmune as well.

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.

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

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 2b 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.

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-

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 3b 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 *vs.* placebo (p=0.121), with all groups without statistically significant results.

4.

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 1year**	**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 by1 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 by1 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
† 1out 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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Ethical approval: Cyprus National bioethics committee (reference ΕΕΒΚ/ΕΠ/2005/10).

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Data Sharing:

No additional unpublished data from the study are available

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 vs placebo on both reducing the annualized relapse rate and disease progression without adverse or significant 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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Figure legends

Figure 1. Omega-6 and omega-3 PUFA, their respective metabolic derivatives and their possible effects on inflammation.

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

Abbreviations: PL, phospholipid; IFN-γ, interferon γ; IL-2, interleukin 2; NFκB, nuclear factor kappa B; PGE2, prostaglandin E2; PPARγ, peroxisome proliferator-activated receptor γ; PUFAs, polyunsaturated fatty acids; TGFβ, 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.

Figure 2. Study Flowchart

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. **

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

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

Panel D demonstrates the dispersion of relapses throughout the 2-year period of all-time on-study (excluding patients on natalizumab) of PLP10 (n=10) *vs.* placebo (n=10). Placebo shows an irregular dispersion of relapses in relation to PLP10 group with linear increasing trend wile PLP10 shows a stabilized linear trend. By using the per-protocol model where patients on natalizumab were excluded, we could compare the number of relapses on a same number of patients.

** Including the patients on natalizumab.

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

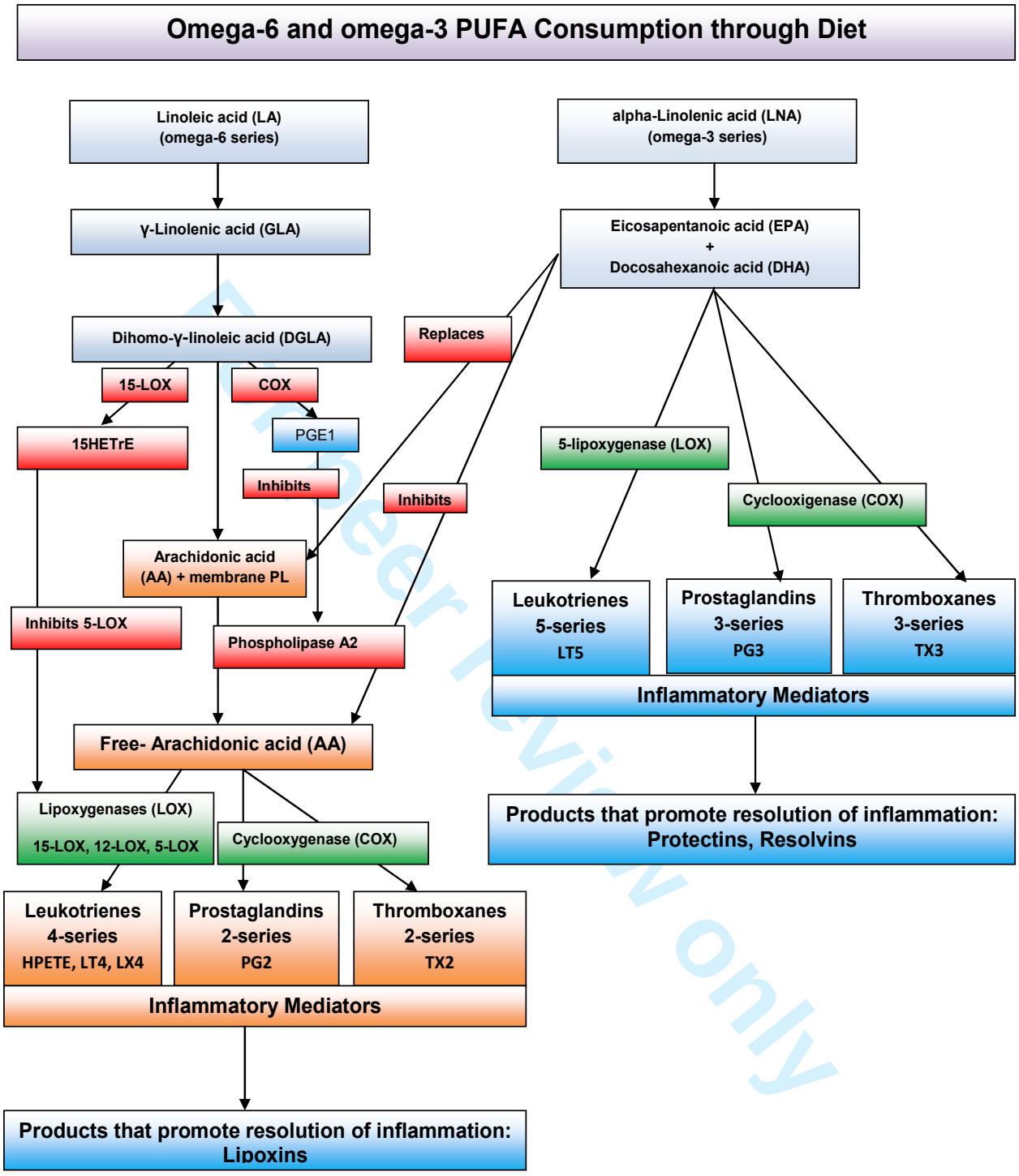
Panel 4B demonstrates the Kaplan–Meier plot of the time to sustained progression of disability among ITT population receiving intervention A, PLP10 and C as compared with placebo. PLP10 reduced the risk of sustained progression of disability by 71% over two years (p=0.052, trend). Intervention formula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

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

¶ Including patients on natalizumab

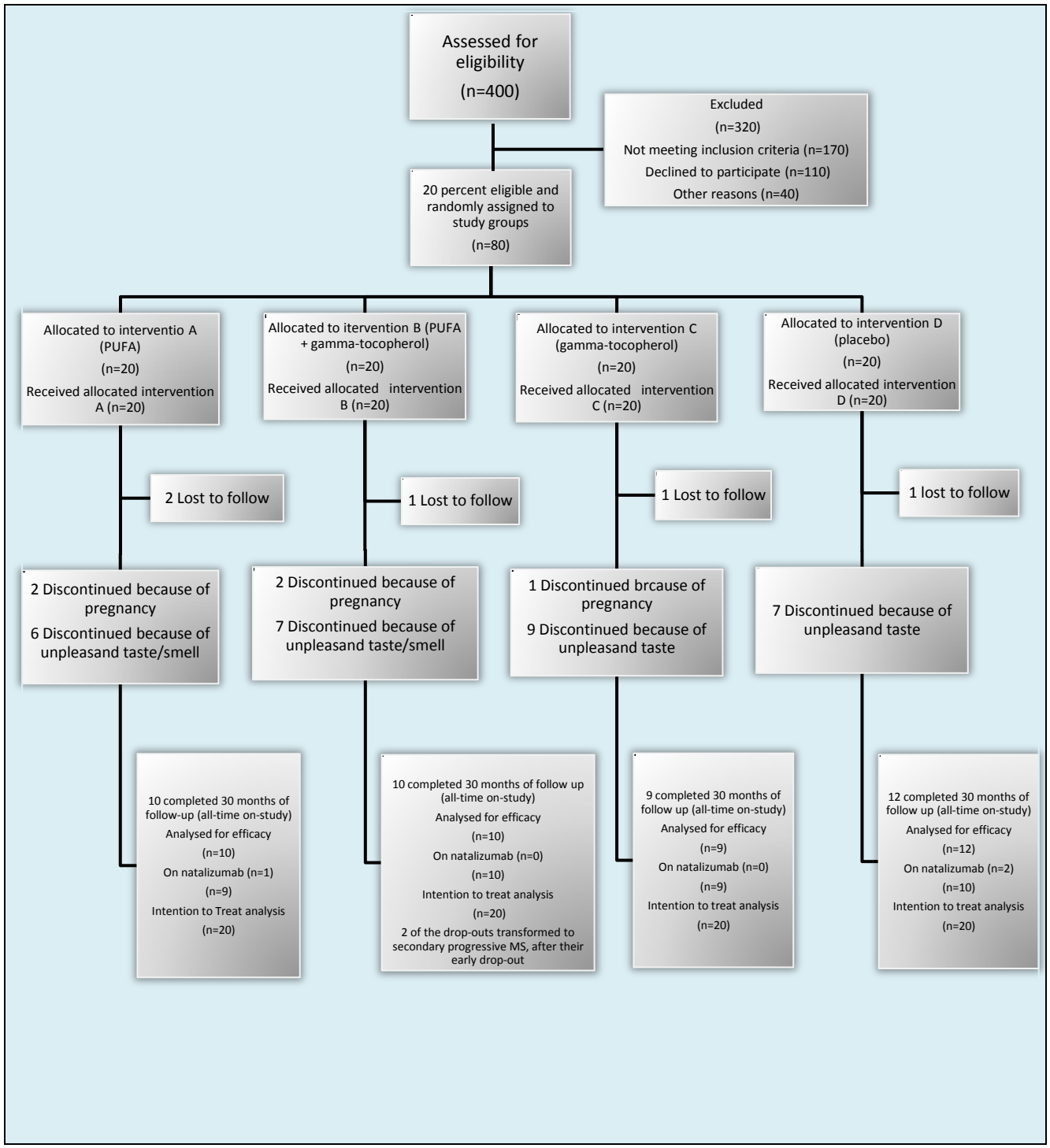
926 ¶¶ Excluding patients on natalizumab

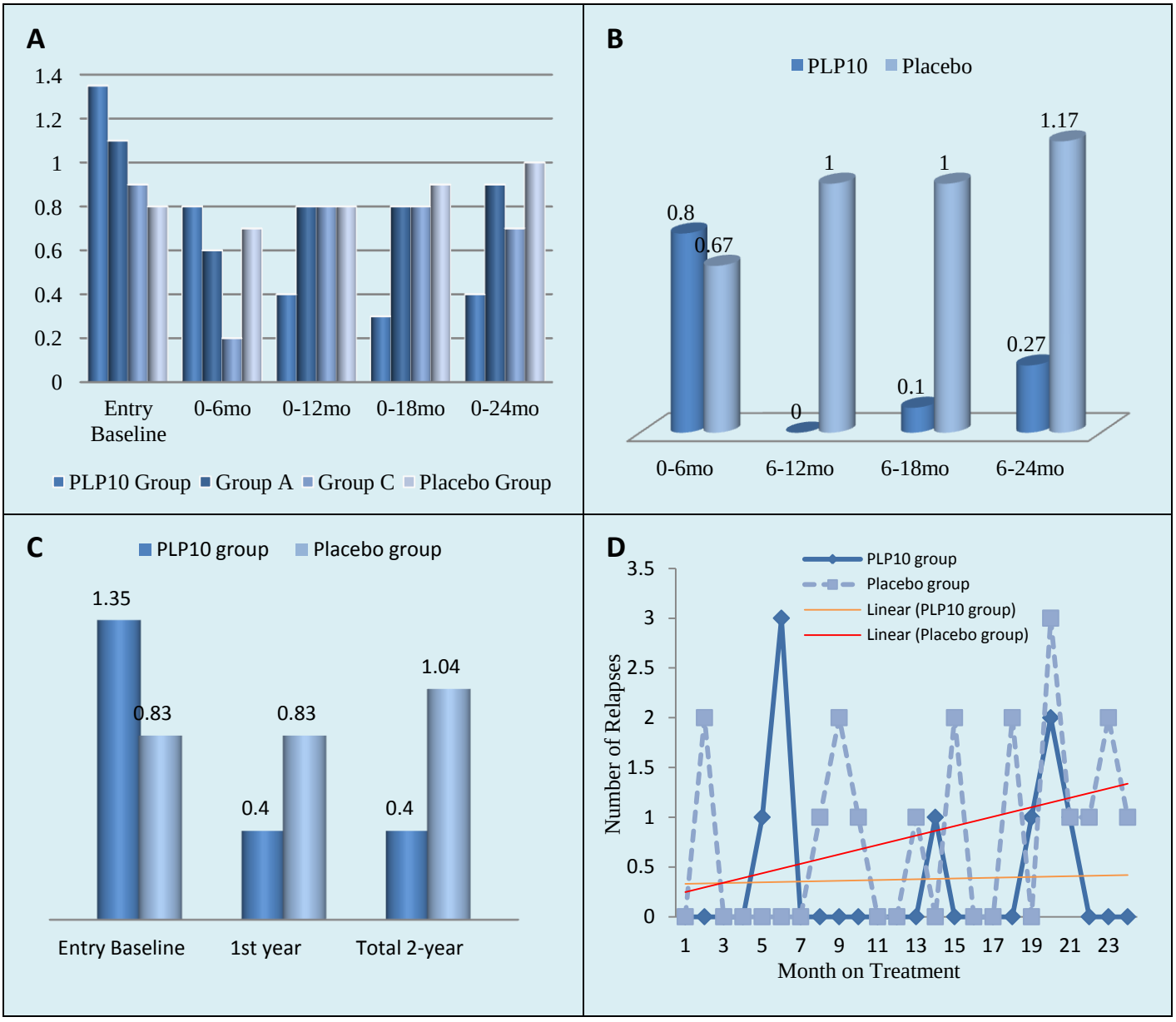
927

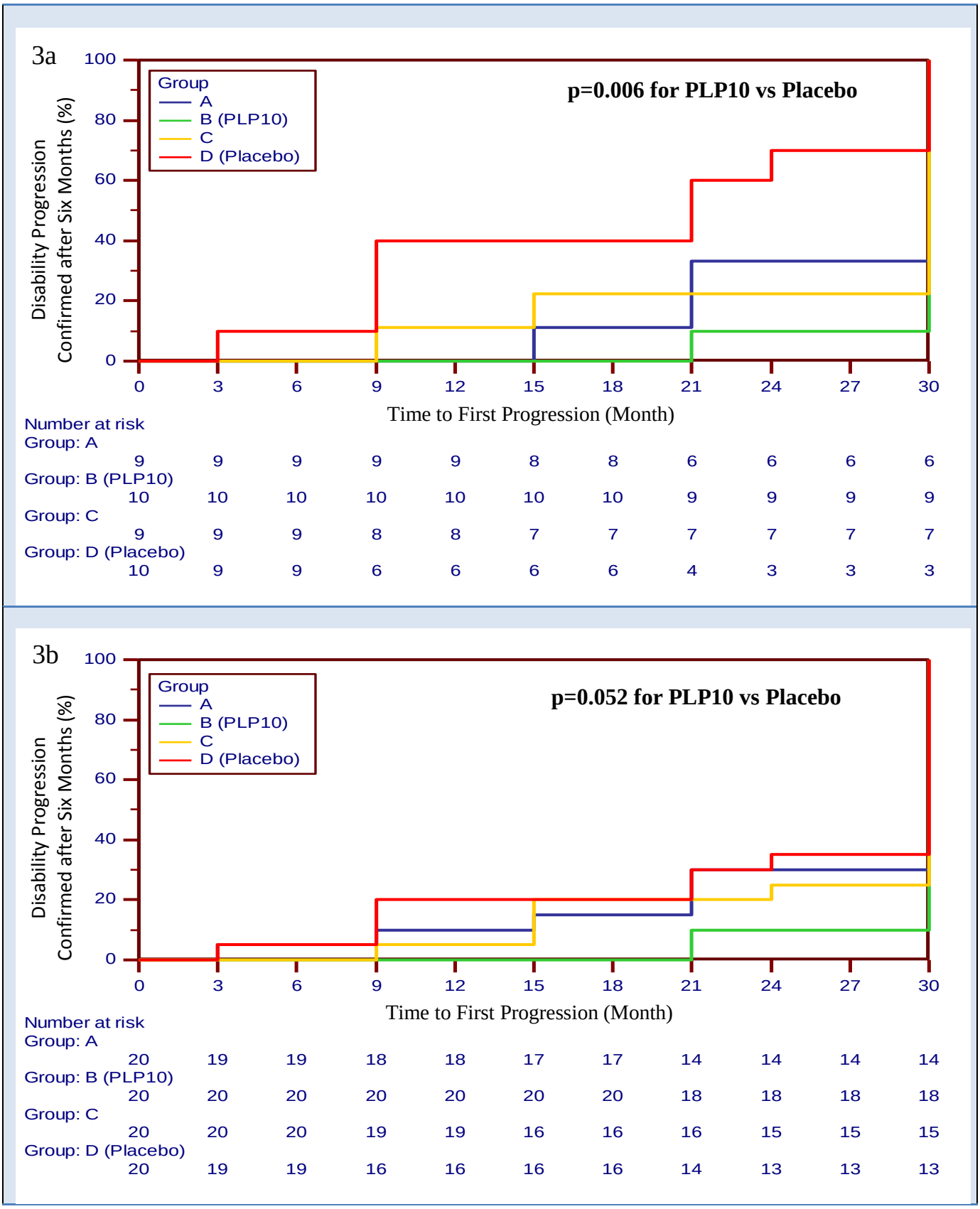


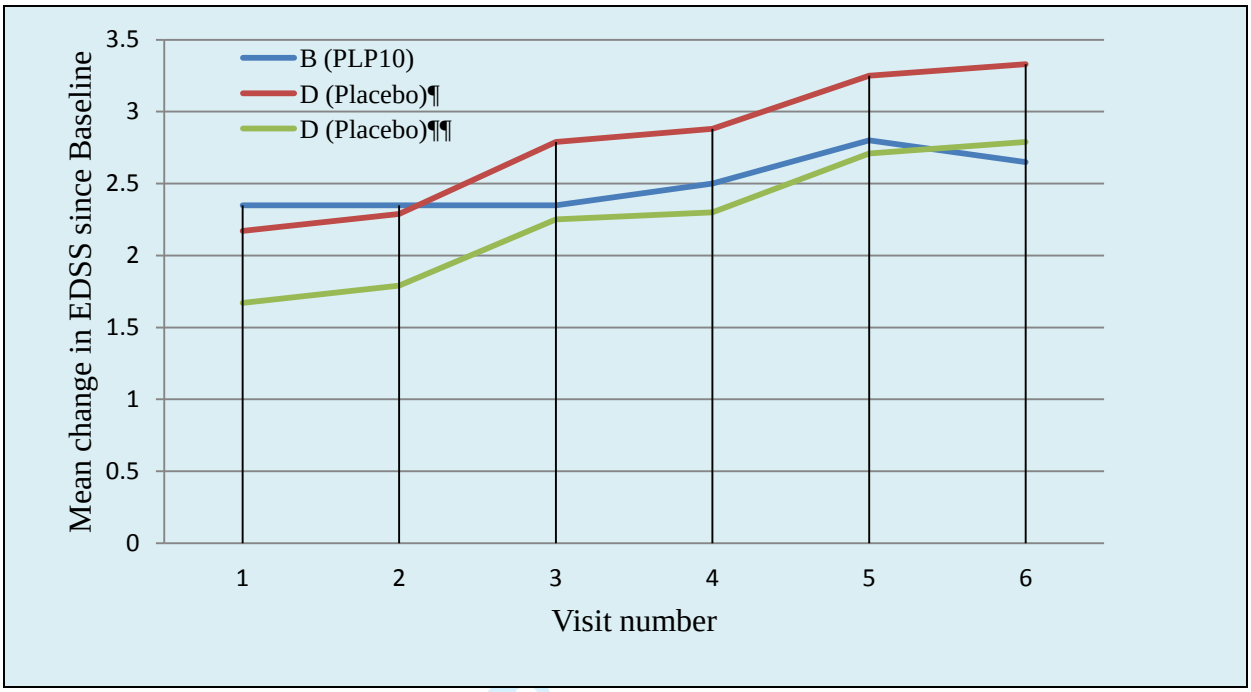
Possible effects on inflammation:

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


Assessed for eligibility
(n=400)
Excluded
(n=320)
Not meeting inclusion criteria (n=170)
Declined to participate (n=110)
Other reasons (n=40)
20 percent eligible and randomly assigned to study groups
(n=80)
Allocated to interventio A (PUFA)
(n=20)
Received allocated intervention A (n=20)
Allocated to itervention B (PUFA + gamma-tocopherol)
(n=20)
Received allocated intervention B (n=20)
Allocated to intervention C (gamma-tocopherol)
(n=20)
Received allocated intervention C (n=20)
Allocated to intervention D (placebo)
(n=20)
Received allocated intervention D (n=20)
2 Lost to follow
1 Lost to follow
1 Lost to follow
1 lost to follow
2 Discontinued because of pregnancy
6 Discontinued because of unpleasand taste/smell
2 Discontinued because of pregnancy
7 Discontinued because of unpleasand taste/smell
1 Discontinued brcause of pregnancy
9 Discontinued because of unpleasand taste
7 Discontinued because of unpleasand taste
10 completed 30 months of follow-up (all-time on-study)
Analysed for efficacy
(n=10)
On natalizumab (n=1)
(n=9)
Intention to Treat analysis
(n=20)
10 completed 30 months of follow up (all-time on-study)
Analysed for efficacy
(n=10)
On natalizumab (n=0)
(n=10)
Intention to treat analysis
(n=20)
2 of the drop-outs transformed to secondary progressive MS, after their early drop-out
9 completed 30 months of follow up (all-time on-study)
Analysed for efficacy
(n=9)
On natalizumab (n=0)
(n=9)
Intention to treat analysis
(n=20)
12 completed 30 months of follow up (all-time on-study)
Analysed for efficacy
(n=12)
On natalizumab (n=2)
(n=10)
Intention to treat analysis
(n=20)


A
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Entry Baseline
0-6mo
0-12mo
0-18mo
0-24mo
PLP10 Group
Group A
Group C
Placebo Group
B
PLP10
Placebo
0.8
0.67
1
0
1
0.1
1.17
0.27
0-6mo
6-12mo
6-18mo
6-24mo
C
PLP10 group
Placebo group
1.35
0.83
0.4
0.83
0.4
1.04
Entry Baseline
1st year
Total 2-year
D
PLP10 group
Placebo group
Linear (PLP10 group)
Linear (Placebo group)
Number of Relapses
3.5
3
2.5
2
1.5
1
0.5
0
1 3 5 7 9 11 13 15 17 19 21 23
Month on Treatment


3a
Group
A
B (PLP10)
C
D (Placebo)
p=0.006 for PLP10 vs Placebo
Disability Progression
Confirmed after Six Months (%)
100
80
60
40
20
0
0 3 6 9 12 15 18 21 24 27 30
Time to First Progression (Month)
Number at risk
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
3b
Group
A
B (PLP10)
C
D (Placebo)
p=0.052 for PLP10 vs Placebo
Disability Progression
Confirmed after Six Months (%)
100
80
60
40
20
0
0 3 6 9 12 15 18 21 24 27 30
Time to First Progression (Month)
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



Supplementary Information

Table of Content

Supplementary Information Methods 1

Intervention and study rational. We are using high dosage of 1:1 omega-3:omega-6 polyunsaturated (PUFA) aiming to overpass and normalize global diet regional traditions and habits in relation to abnormal PUFA/saturated fatty acids (SFA)/monounsaturated fatty acids (MUFA) daily ratio consumption irrespective of the quantities consumed; and enough to equilibrate patients' diet in line with the recommended more physiologic omega-3:omega-6 ratio of about 1:1-4 wt/wt as reported by Simopoulos, 2002. In addition to correct existing deficiencies, cell membrane abnormalities, specifically of the immunopathological system and blood mononuclear peripheral cells, and high enough for availability and immediate ongoing modulation of the involved pathogenic mechanisms and network of events in MS. The high dosage is also required to overpass the quantity limitations, previously discussed, of diet-consumed PUFAs for cellular incorporation, especially in the central nervous system (CNS) of adults. Additionally, fatty acids (FAs) must first cross the intestinal epithelium before reaching the different tissues, where digestion and absorption constitute further problems in their availability (Carlier, H, 1991). Omega-3 PUFA are used in re-esterified form to eliminate unwanted disturbances, at the sides of action, by other fatty acids and molecules present in crude fish oils but also to increase the bioavailability of the FA since triglycerides have been shown to be associated with much higher bioavailability (Dyerberg et al, 2010). Linoleic (LA) and gamma linolenic acid (GLA) are essential structure molecules and important for any physiological (re)generation of cell membrane. GLA quantity is doubled to LA to ensure high direct production of dihomo-gamma-linolenic acid (DGLA), from GLA when LA cannot be metabolized, due to desaturase deficiency or malfunction. Such a reduced capacity to convert LA to GLA has been associated with aging, diabetes, alcoholism, atopic dermatitis, premenstrual syndrome, rheumatoid arthritis, cancer and cardiovascular diseases (Bolton-Smith et al, 1997; Horrobin, 1990; Leventhal et al, 1993). This is going to result in the increase of DGLA relative to arachidonic acid (AA) with DGLA promoting production of prostaglandin (PG)E1 but also inhibition of phospholipase (PL)A2: two major reasons and rational for their use. If other metabolic problems are involved within the omega-6 series and the normal metabolites are not produced then the eicosapentaenoic acid EPA available in PLP10 will substitute the function of DGLA, as a competitive inhibitor of AA for PLA2. In both cases the pro-inflamatory leucotrienes, prostaglandines of the 2-series (PG2) and thromboxanes of the 2 series including the platelet-activator factor (PAF) will be attenuated. The synthesis of AA from DGLA by Δ5 desaturase promoted by LA/GLA supplementation is very limited in humans as a result of limited activity of the enzyme (Yang-Yi & Robert, 1998). AA in the body is mostly available through diet. EPA and docosahexaenoic acid (DHA) are both physiologically important and crucial structured molecules able to substitute excess AA and SFA within the cell membranes. EPA will contribute to the inhibition (competitive to AA) of PLA2, joining the co-supplied omega-6 PUFA but will also participate in the production of anti-inflammatory leukotrienes, prostaglandins of the 3-series (PG3) and thromboxane (TX3) along with DHA, both found in the PLP10 intervention. Moreover EPA will replace AA of the membrane phospholipids and both omega-3 PUFA will contribute replacing abnormal quantities of SFA and excess AA. DHA is used in 3:1 ratio to EPA to cover any possible inabilities of EPA to be metabolized, high enough to strongly promote high production of the aforementioned anti-inflammatory

eicosanoids and cytokines and to be incorporated into CNS cell membranes where DHA should be the major PUFA present, replacing other FA, probably saturated and excess of AA. EPA, DHA, LA and GLA along with the rest of the other ingredients used ("other" omega-3 PUFA, SFA and MUFA that are usually found in the cell membranes of healthy people in limited quantities) in the intervention regimen are for their availability as minor structural constituents of physiological cellular membranes integrity, fluidity and overall function as building blocks for myelin repair and/or myelination. Furthermore the PUFA used within the cocktail intervention aimed to manipulate all other pathophysiological pathways that are reported to be able to: as previously discussed including gene transcription for neuroprotection and remyelination. Furthermore, PUFA are used to ensure the integrity of blood brain barrier (BBB) and to modulate the gelatinases responsible for the T cell migration within the CNS. Three different antioxidant vitamins (vitamin E, mostly as alpha-tocopherol, gamma (γ)-tocopherol (vitamin E isoform) and vitamin A) are used in the regimen preparation to support the cellular antioxidant defenses but also to protect peroxidation of the supplied increased amounts of PUFA. Alpha-tocopherol low-molecular-weight antioxidants will contribute to radical scavenging, interfering with gene transcription, protein expression, enzyme activity and metal chelation (van Meeteren et al, 2005). Vitamin E (alpha tocopherol) and vitamin A are used as antioxidants for the protection of the excess supplemented PUFA, with alpha-tocopherol been demonstrated to protect against peroxynitrite-induced oxidative damage, as well as able to efficiently detoxify hydroxyl, perhydroxyl and superoxide free radicals (the elevated reactive oxygen species (ROS)); each one with different mechanism of action, increasing the effect capability (Vatassery et al, 1998b; van Meeteren, 2005). Gamma-tocopherol is used in high dosage since its half life is very short compared to alpha-tocopherol and has been demonstrated to specifically protect against nitro-radicals. Tocopherols can also exert non-antioxidant properties, including modulation of cell signaling and immune function, regulation of transcription, and induction of apoptosis as previously discussed (van Meeteren et al, 2005).

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

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

adjunct to existing pharmaceuticals produced by reductionism, can be proven therapeutically superior to any available treatment for MS.

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Supplementary Information Methods 2

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

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

The daily intervention formula agent dosages were:

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

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

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

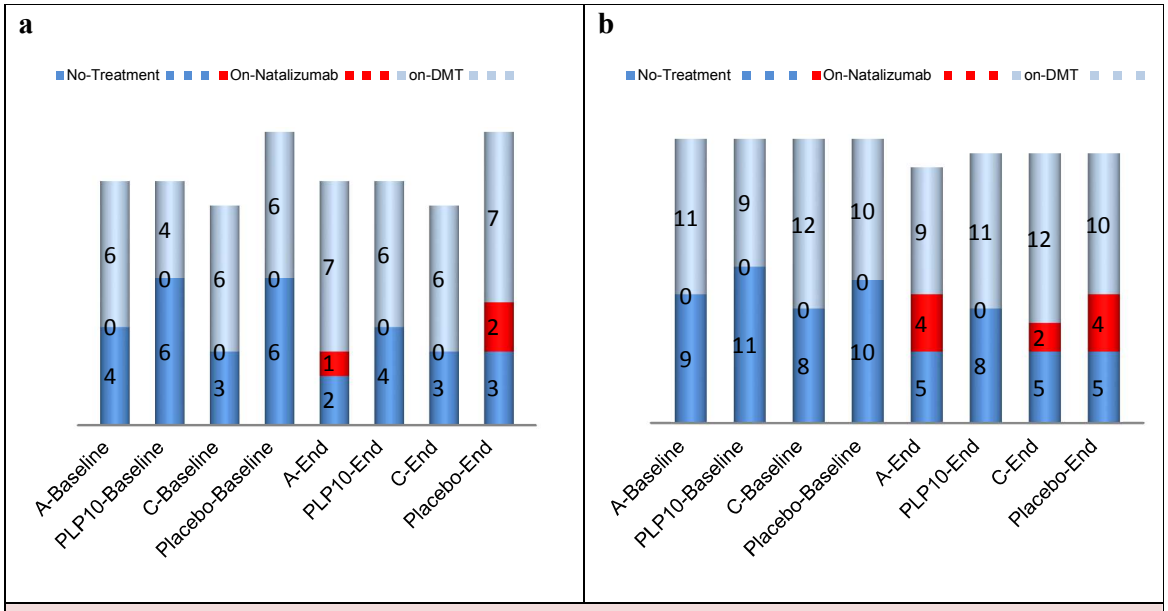
Intervention formula D daily dosage**:** pure virgin olive oil (16930mg).

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

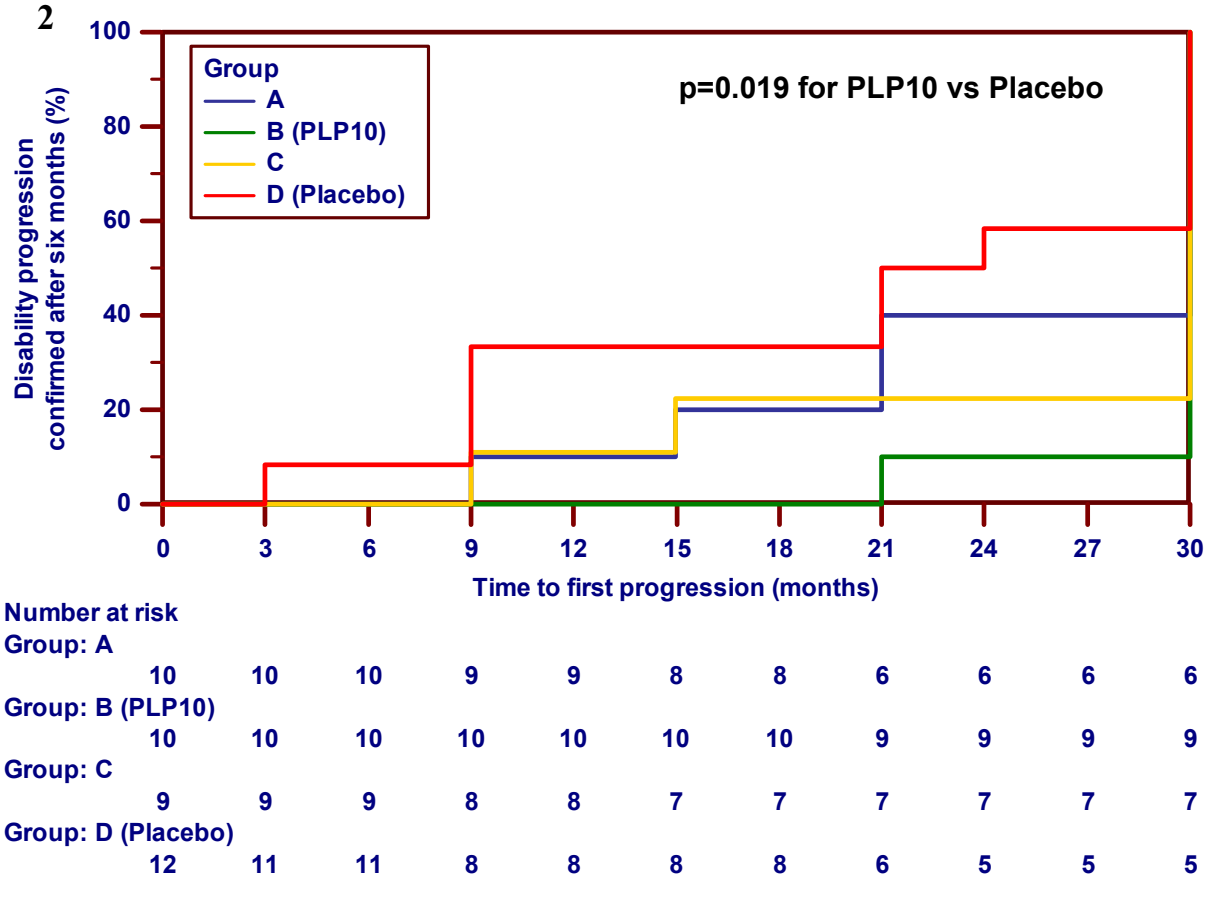
The specific omega-3 related fraction, according to specifications required for the interventions was prepared and purchased from EPAX AS, Aalesund, Norway; as re-esterified glycerides from fish body oils as a source. The specific omega-6 PUFA, MUFA and SFA related fraction, according to required specifications,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

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

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



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.



Supplementary Information Figure 2 | Kaplan–Meier estimates for the time to disability progression. Kaplan–Meier plot of the time to sustained progression of disability among all-time on-study patients, including patients on natalizumab, receiving intervention A, PLP10 and C *vs.* placebo. Intervention PLP10 reduced the risk of sustained progression of disability by 83% over two years (p=0.019). The cumulative probability of progression was 10% in the intervention B group and 58% in the placebo group. Intervention formula A reduced the risk of sustained progression of disability by 32% (p=0.301) and intervention formula C by 62% (p=0.109).

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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

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
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		15,11
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	15,Table 1

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.

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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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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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Keywords: antioxidant vitamins, polyunsaturated fatty acids, multiple sclerosis, systems medicine, randomized clinical trial.

Word Count: 6415

Abstract

Objective To assess whether our three novel interventions, formulated based on systems 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.

Results The per-protocol, proof-of-concept, analysis demonstrated a 64% adjusted relative reduction in ARR at two years for PLP10 versus placebo (P=0.024). Regarding the secondary endpoint, a relative reduction of 86% in the risk of sustained progression of disability was observed within the PLP10 group (p=0.047). No adverse events were reported. Interventions A and C showed no significant efficacy.

Conclusions PLP10 treatment significantly reduced the ARR, and the risk of sustained disability progression without any adverse or severe side effects.

Trial registration International Standard Randomized Controlled Trial, number ISRCTN87818535.

Introduction

Multiple sclerosis (MS) is a complex multifactorial disease that results from interplay between as yet unidentified environmental factors and susceptibility genes.[1-3] Together, these factors trigger a cascade of events, involving engagement of the immune system, inflammatory injury of myelin, axons and glia, functional recovery and structural repair, gliosis, and neurodegeneration.[4] The bio-mechanisms involved are: immune-mediated inflammation, oxidative stress and excitotoxicity.[5-9] These mechanisms may all contribute to oligodendrocyte and neuronal damage and even cell death, hence promoting disease progression. The increasing prevalence of 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.

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

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

Increased or uncontrolled inflammation contributes to several different acute and chronic diseases and it is characterized by the production of inflammatory cytokines, arachidonic acid (AA)–derived eicosanoids (prostaglandins (PGs), thromboxanes (TXs), leukotrienes (LTs), and other oxidized derivatives), other inflammatory agents such as reactive oxygen species (ROS) , nitric oxide (NO), and adhesion molecules (Fig 1).[12] During inflammation glutamate homeostasis is altered by activated immune cells releasing increased quantities of glutamate that can result in over activation of glutamate receptors and in return excitotoxic oligodendroglial death.[7, 13] As such, among others, membrane-related pathology, immune-mediated inflammation, oxidative stress and excitotoxicity provide potentially useful combined targets for intervention in MS.

In vitro and in vivo studies have demonstrated that dietary EPA, DHA, LA, and GLA can be implicated and modulate almost all known complex network of events and pathways repertoire in MS pathophysiology. Brain membrane fatty acid composition can be modified with dietary supplementation, but the process has been showed to be age dependent (it takes much longer in adults versus developing brains) as well as possibly dependent on the quantities of the dietary/supplemented PUFAs.[14] Both human and animal studies proved that diets high in DHA and EPA increase the proportion of these PUFA in the membranes of inflammatory cells and reduce the levels of AA.[12, 15] The anti-inflammatory properties of

omega-3 include production of PGs and TXs of the 3-series and LTs of the 5-series (Fig 1).[14, 16] Resolvins and protectins are biosynthesized from omega-3 fatty acids via cyclooxygenase-2/lipoxygenase (COX-2/LOX) pathways and they promote control of inflammation in neural tissues (Fig 1).[17-21] T-cell proliferation in acute and chronic inflammation can be reduced by supplementation with either omega-6 or omega-3 PUFA.[22] Furthermore, vitamin E is an important antioxidant that can interrupt the propagation of free radical chain reactions.[23] Vitamin E (alpha-tocopherol, an isoform of vitamin E) efficiently detoxifies hydroxyl, perhydroxyl and superoxide free radicals.[24] However γ-tocopherol (another isoform of vitamin E) seems to be more efficiently implicated in trapping NO radicals.[25] In addition alpha–tocopherol exerts non-antioxidant properties, including modulation of cell signaling and immune function, regulation of transcription, and induction of apoptosis.[26]

Moreover, omega-3 fatty acids electrophilic derivatives formed by COX-2, in activated macrophages can stimulate the nuclear respiratory factor (Nrf2) inducing the transcription of neuroprotective and antioxidant related genes, and can activate the peroxisome proliferator-activated receptor (PPAR)γ for anti-inflammatory response.[27-29] In animal studies, EPA and DHA proved to be endogenous ligands of RXRs, with positive effect on neurogenesis.[30] Additionaly, in 2008 Salvati and coworkers reported evidense of accelerated myelination in DHA- and EPA-treated animals.[32] Moreover, DHA and EPA are reported to significantly decrease the levels of metalloproteinases (MMP) -2, -3, -9, and -13 with a significant role in the migration of lymphocytes into the CNS by inducing disruption of the blood brain barrier (BBB), an important step in the formation of MS lesions.[33-39]

Based on the above, specific PUFA and antioxidant vitamins fulfill the criterion of biologic plausibility and have the potential to diminish MS symptoms severity and activity, even

promoting recovery (remyelination).[11] Overall, PLP10 includes multiple ingredients interacting with key interconnected components within functional network modules, each contributing a fraction of the effects of perturbations that cause the disease.[40]

In our phase II, single-center, randomized, double-blind, placebo-controlled, proof-of-concept clinical trial we intended to evaluate the therapeutic ability of PLP10 and of two other interventions (A and C) consisting of PLP10 constituent partial fractions versus placebo, when used on RRMS patients.

Methods

Patients

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

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

The study was conducted in accordance with the standards of the International Conference of Harmonization Guidelines for Good Clinical Practice. The protocol was developed by the investigators and it was approved by the Cyprus National Bioethics Committee and was overseen by an independent safety-monitoring committee evaluating the safety and over-all benefit-risk profiles. The adherence of care providers with the protocol was assessed by an external committee assigned by the funder of the project through reviews of case report forms. All patients gave written informed consent at the time of enrolment.

Randomization and masking

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

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

the conduct of the study were blinded throughout the study. Treating/examining physician, other investigators, pharmacist, neuroradiologist and patients were masked to treatment allocation.

Procedures and end points

The specific omega-3 (re-esterified glycerides) and omega-6 (glycerides) raw materials were purchased according to the required interventions' PUFA-fraction specification (molecular structure, quantity/ratio and quality) with vitamin E (alpha-tocopherol) used as antioxidant stabilizer by the supplier. The vitamins and masking aroma were purchased separately. The mixing of fractions to the final required intervention-composition specification was always performed by the same team of scientists under the supervision of the involved medical biochemist and lipidology specialist, under appropriate conditions every six months. Interventions were stored refrigerated in dark until use. See Table 1 and Supplementary Information Methods 1 and 2 for intervention specification detailed description and study/intervention rational.

Participants were randomly assigned to receive a daily dose of a mixture of 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 A, group A); or composed mixture of pure γ-tocopherol (760mg) dispersed in pure virgin olive oil (16,137 mg) as delivery vehicle (intervention C, group C); or a mixture of intervention formula A with intervention C without the pure virgin olive oil (intervention B, named PLP10, group B); or placebo composed of pure virgin olive oil (16,930mg) (intervention D, group D) (Table 1). 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. The institution's pharmacist was responsible for the appropriate storage and handling of the interventions to the individual participants. The interventions were taken orally once daily 30 minutes before dinner by a dosage calibrated cup for 30 months. The ingredients, ratio and dose have been selected based on their biophysical interrelation to the total known multiple MS causing factors, their biochemical importance and the role expected to play in the normalisation and treatment of the involved complex network of events in the disease pathophysiology. Moreover, the high intake dosage was used to overcome any abnormal dietary accumulation of related agents as a result of patients' food intake habits, irrespective of geographical origin, in relation to the daily consumption ratio of the total fatty acid intake; in order to end-up with omega-3 to omega-6 PUFA indicated physiological body ratio composition of 1:1 wt/wt.

The period beginning from July 1st 2007 (enrolment) until December 31st 2007 (entry baseline) was used for normalization period. This six-month normalization period would allow the interventions' agents to exert their beneficial effect (for the incorporation/normalization of cell membranes by oral PUFA, since they need four to six months to exert pivotal action on immune and neural cells, correction of antioxidant deficiency and body PUFA, and optimal normalization of EPA and DHA levels/ratio).[41-43] The study was completed on December 31st 2009 and the recording of relapses continued until December 31st 2010. More clearly the study included the "normalization period" (July 1st 2007 to Dec 31st 2007), the "on treatment" period (Jan 1st 2008 to Dec 31st 2009) and the 12-month "extended period" (Jan 1st 2010– Dec 31st 2010).

Depending on their clinical status and in accordance with the ethical issues governing clinical trials participants continued receiving the indicative regular available treatments, according to

international guidelines with persistent evaluation of any side-effects and adverse events. The study was designed to end 30 months after enrolment and clinical assessments were scheduled at entry baseline, 3, 9, 15, 21 and 24 months on-treatment. Patients were also clinically examined by the treating neurologist within 48 hours after the onset of new or recurrent neurologic symptoms.

The primary end point was the ARR at two years. A relapse was defined as new or recurrent neurologic symptoms not associated with fever or infection that lasted for at least 24 hours and accompanied by new neurologic signs. Relapses were treated with methyl-prednisolone at a dose of 1g intravenous per day, for three days followed by prednisone orally at a dose of 1mg/kg of weight per day on a tapering scheme for three weeks. The secondary end point at two years was the time to confirmed disability progression, defined as an increase of 1.0 or more on EDSS, confirmed after six months (progression could not be confirmed during a relapse). The final EDSS score was confirmed six months after the end of the study. A post-hoc analysis was performed assessing the proportion of patients free from new or enlarging T2 lesions on brain MRI scans at the end of the study for the per-protocol participants of the group receiving the highest effective intervention versus placebo. Comparison was made only versus the available archival MRI scans up to three months before the enrolment date. MRI scans were performed and blindly analyzed at an MRI evaluation centre. The patients continued to be followed for additional 12 months after completion of the trial and relapses were recorded. Finally, patients were strongly encouraged to remain in the study for follow-up assessments even if they had discontinued the study drug.

Blood samples were collected from all randomized patients at the time of enrolment, at every scheduled clinical assessment and during relapses. To check individual compliance with

intake, the fatty acids composition of patients' red blood cells' membranes was determined, by gas chromatography, according to a standard protocol. The fatty acid analyses were performed after study termination and thus did not influence the blinding.

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

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

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

Statistical analysis

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

Baseline characteristics were compared across all intervention groups by ANOVA or Kruskal-Wallis rank test for continuous variables and by mean Fisher's exact test for categorical variables, as appropriate.

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

For the secondary end-point outcome, the time to disability progression, Kaplan–Meier curves were constructed. Progression to disability and time thereof was compared in a pair-wise fashion for the active interventions versus placebo by the log-rank test in the main analysis and by Cox proportional-hazards models with adjustment for baseline EDSS score, age and DMT in the supportive analysis. Each test was performed with a significance level of 0.05. Multivariate models considered all variables with $P < 0.1$ on univariate models. There was no overt violation of the proportionality assumption.

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

Role of the funding source

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

Results

Study population

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

Among the 80 patients, 20 patients were randomly assigned to each of the three groups to receive the interventions and 20 to receive placebo (Fig 2). Baseline characteristics of both the ITT and the per-protocol populations were similar across groups (Table 2A and 2B). Total drop-out patients completed follow-up until study completion and were included in the ITT analyses (Table 4). Five patients were totally lost to follow before their first scheduled visit and two patients dropped-out before their first scheduled visit progressed to secondary progressive MS. Fifteen patients dropped-out without successfully completing the "normalization" period including five pregnancies. Another 17 patients dropped-out early after entry baseline. Seven patients that dropped out were given monoclonal antibody treatment (natalizumab). Overall, a total of 41 (51%) patients completed the 42-month study (July 2007 through December 31st 2010, including the 12-month extended period) where one patient from group A and two from the placebo group transferred on natalizumab, and 39 (49%) patients either withdrew (drop-out) or lost to follow. Reasons for study-interventions discontinuation are listed in Figure 2.

Efficacy

Relapses

As a proof-of-concept trial we primarily needed to answer whether the interventions were effective for those MS patients who adhere to the assigned treatment, the per-protocol analysis.[45] For the sake of methodological comprehensiveness we also present the ITT analysis as a secondary analysis, to answer a different question, complementary to our core hypothesis; like what happened to MS patients who were placed on the interventions (the effect of assignment).[45] Otherwise, as a result of a high drop-out rate, an ITT analysis will not likely be able to show the superiority of an intervention even if it is effective.[45] In any instance, the proper approach of evaluating a study data is to understand what question

prompted the research and assure that the analysis is appropriate for providing the answer whatever it is called. Both analyses can be performed for a study, using the results from the different analyses to answer different research questions.[45] These interventions are original, composed by a different treatment rational, the SM, never tested before and the important main concern was to evaluate their efficacy and safety based on the per-protocol treated MS patients, without any peripheral noise. The question that had to be answered was: "what happens to the patients that are placed and stick on the specific treatment".

Regarding the per-protocol analysis, during the first year of treatment, the ARR was 0.80, 0.40, 0.78 and 0.83 for the four intervention groups respectively. During the second year, the ARR was 0.90, 0.40, 0.67 and 1.25 for the four intervention groups respectively. Overall, for the two-year primary end point, 8 relapses were recorded for the 10 patients in PLP10 group (0.40 ARR) versus 25 relapses for the 12 patients in placebo (1.04 ARR), a 64% adjusted relative rate reduction (RRR) for the PLP10 group (RRR 0.36, 95% confidence interval (CI) 0.15 to 0.87, p=0.024) (Tables 3A, 5 and Fig 3A and 3C). Excluding 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, Tables 3B and 5). Pair-wise comparisons for the other two groups against placebo did not yield statistically significant results (Tables 3A, 3B). The proportion of patients with ≤1 relapse for the two years on-study was higher in the PLP10 group than in the placebo group (90% versus 42%, p=0.030, Table 5). Seeking to investigate further the observed difference, we compared the relapse rate during the 24 months before entry to the study to the 24 months on-treatment for each intervention group. We observed a statistically significant relative reduction in the ARR (70%) only in the PLP10 group (RRR 0.30; 95% CI 0.14 to 0.65, p=0.003, Table 3A); within-group comparisons for the three other groups ARR reduction was not significant and

remained not significant when natalizumab treated patients were further excluded from the analysis. The effect of PLP10 through time at different time-windows versus placebo for all-time on-study patients is shown in Figures 3A to 3D. The ARR analysis, within time-windows, was not an assigned endpoint, but it could help in the process of evaluating parallel information as the time needed for a specific treatment intervention activity to be evident, as well as the efficacy profile through time. PLP10 reached maximum effect within a year on-treatment (counting from the entry baseline) and remained stable at an ARR of 0.4, displaying a steadily reduced ARR with long free-relapse time-windows. These group B characteristics are considered important parameters of a successful MS treatment where the rule than the exception is the heterogeneity among patients' disease evolution. Specifically, Figure 3D demonstrates the dispersion of relapses throughout the 2-year period of all-time on-study (excluding patients on natalizumab) of PLP10 (n=10) versus placebo (n=10). Placebo, in line with the existing knowledge of how relapse history works in relation to future relapses on MS patients (contagion phenomenon) indicates the expected linearly increased trend of the relapse incidence.[46] The same phenomenon was true for the groups A and C. Finally, during the 12 month post-study extended period (January 1st 2010 to December 31st 2010) all-time on-study patients that received PLP10, showed persistent benefit in the ARR compared to placebo (six relapses for the 10 subjects within PLP10, 0.6 ARR versus19 for the 12 subjects within placebo group, 1.58 ARR) indicating a statistically significant 62% adjusted relative rate reduction in the ARR for the PLP10 group (RRR 0.38, 95% CI 0.12 to 0.99, p=0.046).

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

patients within the placebo arm population were on natalizumab, including the two patients that transferred while all-time on-study versus none within PLP10 group (Supplementary 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). 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 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 patients on natalizumab, there was an increased statistically significant difference between the PLP10 and the placebo group for the same analysis (p=0.006) (Fig 4A). At two years, the cumulative probability of progression was 10% in the PLP10 group and 70% in the placebo group, which represents a decrease of 60 percentage points or a relative 86% decrease in the risk of sustained progression of disability within 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 difference was observed for any comparison of the other two groups compared to placebo (Fig 4A and Supplementary Information Fig 2).

Regarding the ITT analysis, at two years, the cumulative probability of progression was 10% in the PLP10 group and 35% in the placebo group (p=0.052, a trend for an effect), which represents a decrease of 25 percentage points or a relative 71% decrease of the PLP10 for the risk of sustained progression of disability (adjusted hazard ratio 0.22, 95% CI 0.04 to 1.07, p=0.06) (Fig 4B). Two versus seven out of the total randomized patients progressed to confirmed disability in the PLP10 and the placebo groups respectively. No significant differences were observed for groups A or C against placebo (Fig 4B). The mean change in Expanded Disability Status Scale (EDSS) score as a function of visit number is shown in Figure 5.

MRI

Over two years, the MRI results support the overall conclusion from the study that PLP10 has a positive effect on disease activity since only 29% from the PLP10 group as opposed to 67% from the placebo group developed new or enlarging T2 lesions (57% relative risk reduction). Excluding patients on natalizumab there is an increased relative risk reduction (64%) between PLP10 as opposed to placebo with 29% of patients on PLP10 and 80% on placebo with development of new or enlarging T2 lesions (Table 5).

Safety

Over the course of the 30 month study no significant adverse events were reported from any group. According to a questioner procedure the only aetiology for drop-outs was the palatability and smell of the formula preparations. Nausea was reported by two patients. No

abnormal values observed on any of the biochemical and haematological blood tests. No allergic reactions reported.

Discussion

In this proof-of-concept clinical trial assessing the safety and efficacy of three cocktail intervention formulas in RRMS, we observed a significant benefit for the novel PLP10 intervention compared to placebo for both the ARR and the progression to disability. Our results include analyses pertaining to a total of 42 months study collected data, including the 12-month, free of intervention treatment, extension period. We focused on the per-protocol data analysis since it is the appropriate method to best provide the answer to the proof-of-concept trial-addressed question. The high drop-out rate was solely the result of formulas palatability, a common phenomenon in trials using oily interventions where a lot of patients tend to drop-out soon after first dosage. We thus present our main per-protocol analysis, as well as a subgroup analysis excluding patients on natalizumab. We have found a statistically significant reduction in the ARR and the disability progression comparing not only patients on PLP10 versus placebo but also comparing the ARR of the PLP10 patients in the 24-month period prior to the study to the ARR of the 24 months on-study; the observed differences became larger when patients that received natalizumab (the most potent disease modifier) were excluded. The ARR decreased within a year on PLP10 and significantly remained stable until study completion. Statistically significant difference of ARR between patients on PLP10 versus placebo continued for the additional 12 month extended period (persistent effect) without significant difference on DMT. These clinical findings are supported by the results regarding the MRI analysis where the proportion of patients free from new or enlarging brain T2 lesions was also higher in PLP10 group versus placebo. The persistent effect within the extended period it is considered of major importance and supportive of the results since it is

in agreement with the very long washouts, reported necessary, for omega-3 fatty acids and especially DHA to return towards pretreatment values within the fatty acids of plasma, platelets, monocytes and red blood cells.[42] This study also provides important 30-month, placebo-controlled information about the safety of PLP10, A and C interventions, where no any adverse or severe side effects have been reported.

As medications used to treat MS become increasingly highly specific and potent, attention to safety is paramount. Current available treatments are products of reductionism, partially effective, associated with severe side effects without (re)myelinating or neuroprotective abilities. Interferons and glatiramer acetate, the most widely used first-line MS drugs available today, are associated with the least severe side-effects among MS therapies but they are reported with only 29-33% ARR reduction and with no significant effects on the progression of disability. Natalizumab as previously discussed and Fingolimod with 54% ARR reduction (without significant benefit on the progression of disability) are second-line drugs associated with severe side-effects.[47, 48]

No existing MS treatment has ever been designed as a result of SM concept approach or with a potential to effectively stimulate intrinsic remyelinating and neuroprotecting mechanisms or exert such an action. Now we propose that a holistic SM model approach has to be applied by synchronized action on all involved perturbed mechanisms. PLP10 has innovative characteristics like no any other intervention or medication tried before for MS treatment, with unique efficacy abilities through different mechanisms of action, probably by the synergistic effect of its constituent ingredients. PLP10 has all the characteristics of a medical food with the action to feed a normal metabolic process by supplying nutritional structural membrane precursors, building blocks, and vitamins from dietary sources that enhance

remyelination and neuroprotection and simultaneously promote normalization of all cellular membranes lipid content. The intention is to normalize the specific nutritional requirements of the MS patients.

Different factors and molecular entities appear to be part of the possible aetiology for MS with specific PUFA and antioxidants found to be key substances related to all known pathogenic and recovery mechanisms. But, it is well established that MS patients are characterized with significant deficiencies in antioxidant efficiency as well as in PUFA levels in blood and cellular membranes.[11, 49-51]

According to one hypothesis, the change in the ratio of omega-3 to omega-6, due to increasing consumption of omega-6 PUFA, meaning high accumulation of AA, in the Western diet, may be one of the major factors responsible for the increasing incidence of inflammatory diseases relative to populations. In the Western diet, the ratio of omega-3 to omega-6 is about 1:20–30; in populations that consume fish-based diets, the ratio is about 1:1–2.[52, 53] The intervention daily dose was aiming and believed to be high enough to restore/amplify body efficient antioxidant activity and ensure cellular membranes lipid profile normalization (PUFA content) and simultaneously potentiate involvement of the ingredients in the anti-inflammatory and recovery mechanisms. Diet fatty acid molecules need about six months period to exert their beneficial effect and this essential parameter was for the first time under consideration in our study design (normalization period).[42] This chronotherapy parameter it is of major importance in line with the SM treatment philosophy and if it is not included in the trial design the possibility of misleading result evaluation greatly increases. In fact, considering that omega-3 supplementation can release and replace excess AA within the

cellular membranes, we can speculate that an increased inflammatory activity can possibly result during the first six months of supplementation (during normalization period).

The maintenance of myelin requires continued turnover of its components throughout life.[54,55] In addition to the EPA, DHA, LA, and GLA, PLP10 was containing limited quantities of other structural PUFA, specific MUFA (mostly oleic acid) and SFA (palmitic and stearic acid), specifically to provide a direct source for neuronal cell membranes rehabilitation and for (re)myelination and neuroprotection since they are all major components, precursors and building blocks of any new physiological myelin and cellular membranes in general. Assembly of the correct molecules into myelin membrane may be especially critical during active synthesis. Possibly, if critical constituents aren't available or are metabolically blocked, amyelination, dysmyelination or demyelination may ensue.[56]

The well known and established safety of the ingredients used and the protocol guidelines were supportive reasons for us to proceed with the clinical study even though with limitation on the pre-estimation of required trial sample size as it was discussed in method section. The adherence of the subjects is another issue but the duration of the study (42 months) is adding power to the results;[44] having the research questions been consciously and carefully approached and answered. Furthermore, the statistical methodologies used along with the appropriate adjustments, broadly accepted for MS clinical trials, power the findings, results, and significance. The baseline characteristics of the treatment arms could possibly be considered indicative of four very active groups of patients but that was the result of the limited number of RRMS population eligible for the study within Cyprus. On the other hand the balanced baseline characteristics without statistical differences, the statistical adjustments (for all important baseline parameters i.e. relapses, EDSS, age and DMT) and the

randomization within four different groups are the safety valves against data misinterpretation. Yet, in small randomized control trials with a high drop-out rate, the per-protocol analysis could be affected by the characteristics of the patients dropping out. In order to safeguard our findings in the best possible way under the circumstances, we proceeded to adjusting for confounders. Moreover, we cannot discard our finding as a false positive, given that this is a randomized, double-blind, placebo-controlled clinical trial and, despite its small sample size, represents a piece of evidence that only a larger randomized controlled trial can replicate or refute. It is possible to question why DMTs efficacy cannot be emerged out of the data analysis, of the four treatment arms, and in accordance to their published values. We believe that the limited efficacy of the DMTs, the sample size and the statistical adjustments were strong limiting determining factors for such an indication to be countable. An additional argument is that the efficacy reported for the analysis of pre-treatment (24 months before entry baseline) versus on-trial ARR could be considered as potentially biased due to differences of how relapses were defined during the course of a study compared to pre-treatment period; or due to regression to the mean or placebo effect. This analysis was performed as an additional exploratory analysis that we were able to do due to the availability of data. The relapses of the two pre-treatment years were drawn out of the patients' archival records by the same treating neurologist involved in the study (MP), and according to the patients' hospitalization date for receiving intravenous methyl-prednisolone. This analysis was not used as a primary or a secondary end-point under investigation although it is usually reported by many clinical studies. As a matter of fact many early phase trials are based only on such an analysis (before versus after treatment results). In almost all MS trials the number of relapses within the two years before baseline is a factor under adjustment for the statistical analyses.[48] The inclusion of the post-hoc MRI analysis is another limiting factor that needs attention since it was used as an additional aside exploratory

approach (due to study budget limitations it was not possible to be used as a formal endpoint); but the MRI evaluation was blinded and can be considered as representative of the randomized subjects within the treatment arms. As far as the regression to the mean and the placebo effect concerns we believe that the 6-month normalization period is an accountable and valuable eliminating factor of the possible effect; as well as the presence of four groups, where only the PLP10 treatment arm is associated with statistically significant efficacy versus placebo. It is a placebo-controlled study after all.

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

It is commonly accepted that nutrition is one of the possible environmental factors involved in the pathogenesis of MS, but its role as complementary MS treatment is unclear and largely disregarded.[57] It is well known that the majority of the patients suffering from MS they do use dietary supplements for a variable length of time and they prefer supplement type of

"help" over conventional drugs.[58] Dietary antioxidants and fatty acids may influence the disease process in MS by reducing immune-mediated inflammation, oxidative stress and excitotoxic damage.[11] Present data reveal that healthy dietary molecules have a pleiotropic role and are able to change cell metabolism from anabolism to catabolism and down-regulate inflammation by interacting with enzymes, nuclear receptors and transcriptional factors.[57] The present study, for the first time provides strong link evidence between dietary, metabolic, immunological, and neurobiological aspects of MS after three quarters of a century of unsuccessful scientific efforts. This might probably be the beginning of opening new horizons and new avenues in the approach of MS prevention and treatment, and possibly of other multifactorial chronic diseases, including neurodegenerative and autoimmune as well.

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.

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.

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

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.

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.

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 1year**	**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 by1 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 by1 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
† 1out 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 for the protocol and study design. M.C.P was the evaluator of patients' clinical progress signs and the treatment physician. I.S.P and G.N.L were involved in the non-pharmacologic treatment-related care. I.S.P performed the literature search and both I.S.P and G.N.L contributed on the intervention formulation and composition rational. I.S.P supervised the composition procedure of the interventions and the fatty acid profile analysis of the red blood cell membranes. E.E.N and I.S.P performed the statistical analyses. E.E.N was an independent scientist. All authors vouch for the accuracy and completeness of the data and the statistical analyses.

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Ethical approval: Cyprus National bioethics committee (reference ΕΕΒΚ/ΕΠ/2005/10).

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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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Figure legends

Figure 1. Omega-6 and omega-3 PUFA, their respective metabolic derivatives and their possible effects on inflammation.

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

Abbreviations: PL, phospholipid; IFN-γ, interferon γ; IL-2, interleukin 2; NFκB, nuclear factor kappa B; PGE2, prostaglandin E2; PPARγ, peroxisome proliferator-activated receptor γ; PUFAs, polyunsaturated fatty acids; TGFβ, 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.

Figure 2. Study Flowchart

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. **

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

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

Panel D demonstrates the dispersion of relapses throughout the 2-year period of all-time on-study (excluding patients on natalizumab) of PLP10 (n=10) *vs.* placebo (n=10). Placebo

shows an irregular dispersion of relapses in relation to PLP10 group with linear increasing trend wile PLP10 shows a stabilized linear trend. By using the per-protocol model where patients on natalizumab were excluded, we could compare the number of relapses on a same number of patients.

** Including the patients on natalizumab.

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

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

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

¶ Including patients on natalizumab

¶¶ Excluding patients on natalizumab

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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Keywords: antioxidant vitamins, polyunsaturated fatty acids, multiple sclerosis, systems medicine, randomized clinical trial.

Word Count: 6415

Abstract

Objective To assess whether our three novel interventions, formulated based on systems 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.

Results The per-protocol, proof-of-concept, analysis demonstrated a 64% adjusted relative reduction in ARR at two years for PLP10 versus placebo (P=0.024). Regarding the secondary endpoint, a relative reduction of 86% in the risk of sustained progression of disability was observed within the PLP10 group (p=0.047). No adverse events were reported. Interventions A and C showed no significant efficacy.

Conclusions PLP10 treatment significantly reduced the ARR, and the risk of sustained disability progression without any adverse or severe side effects.

Trial registration International Standard Randomized Controlled Trial, number ISRCTN87818535.

Introduction

Multiple sclerosis (MS) is a complex multifactorial disease that results from interplay between as yet unidentified environmental factors and susceptibility genes.[1-3] Together, these factors trigger a cascade of events, involving engagement of the immune system, inflammatory injury of myelin, axons and glia, functional recovery and structural repair, gliosis, and neurodegeneration.[4] The bio-mechanisms involved are: immune-mediated inflammation, oxidative stress and excitotoxicity.[5-9] These mechanisms may all contribute to oligodendrocyte and neuronal damage and even cell death, hence promoting disease progression. The increasing prevalence of 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.

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

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

Increased or uncontrolled inflammation contributes to several different acute and chronic diseases and it is characterized by the production of inflammatory cytokines, arachidonic acid (AA)–derived eicosanoids (prostaglandins (PGs), thromboxanes (TXs), leukotrienes (LTs), and other oxidized derivatives), other inflammatory agents such as reactive oxygen species (ROS) , nitric oxide (NO), and adhesion molecules (Fig 1).[12] During inflammation glutamate homeostasis is altered by activated immune cells releasing increased quantities of glutamate that can result in over activation of glutamate receptors and in return excitotoxic oligodendroglial death.[7, 13] As such, among others, membrane-related pathology, immune-mediated inflammation, oxidative stress and excitotoxicity provide potentially useful combined targets for intervention in MS.

In vitro and in vivo studies have demonstrated that dietary EPA, DHA, LA, and GLA can be implicated and modulate almost all known complex network of events and pathways repertoire in MS pathophysiology. Brain membrane fatty acid composition can be modified with dietary supplementation, but the process has been showed to be age dependent (it takes much longer in adults versus developing brains) as well as possibly dependent on the quantities of the dietary/supplemented PUFAs.[14] Both human and animal studies proved that diets high in DHA and EPA increase the proportion of these PUFA in the membranes of inflammatory cells and reduce the levels of AA.[12, 15] The anti-inflammatory properties of

omega-3 include production of PGs and TXs of the 3-series and LTs of the 5-series (Fig 1).[14, 16] Resolvins and protectins are biosynthesized from omega-3 fatty acids via cyclooxygenase-2/lipoxygenase (COX-2/LOX) pathways and they promote control of inflammation in neural tissues (Fig 1).[17-21] T-cell proliferation in acute and chronic inflammation can be reduced by supplementation with either omega-6 or omega-3 PUFA.[22] Furthermore, vitamin E is an important antioxidant that can interrupt the propagation of free radical chain reactions.[23] Vitamin E (alpha-tocopherol, an isoform of vitamin E) efficiently detoxifies hydroxyl, perhydroxyl and superoxide free radicals.[24] However γ-tocopherol (another isoform of vitamin E) seems to be more efficiently implicated in trapping NO radicals.[25] In addition alpha–tocopherol exerts non-antioxidant properties, including modulation of cell signaling and immune function, regulation of transcription, and induction of apoptosis.[26]

Moreover, omega-3 fatty acids electrophilic derivatives formed by COX-2, in activated macrophages can stimulate the nuclear respiratory factor (Nrf2) inducing the transcription of neuroprotective and antioxidant related genes, and can activate the peroxisome proliferator-activated receptor (PPAR)γ for anti-inflammatory response.[27-29] In animal studies, EPA and DHA proved to be endogenous ligands of RXRs, with positive effect on neurogenesis.[30] Additionaly, in 2008 Salvati and coworkers reported evidense of accelerated myelination in DHA- and EPA-treated animals.[32] Moreover, DHA and EPA are reported to significantly decrease the levels of metalloproteinases (MMP) -2, -3, -9, and -13 with a significant role in the migration of lymphocytes into the CNS by inducing disruption of the blood brain barrier (BBB), an important step in the formation of MS lesions.[33-39]

Based on the above, specific PUFA and antioxidant vitamins fulfill the criterion of biologic plausibility and have the potential to diminish MS symptoms severity and activity, even

promoting recovery (remyelination).[11] Overall, PLP10 includes multiple ingredients interacting with key interconnected components within functional network modules, each contributing a fraction of the effects of perturbations that cause the disease.[40]

In our phase II, single-center, randomized, double-blind, placebo-controlled, proof-of-concept clinical trial we intended to evaluate the therapeutic ability of PLP10 and of two other interventions (A and C) consisting of PLP10 constituent partial fractions versus placebo, when used on RRMS patients.

Methods

Patients

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

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

The study was conducted in accordance with the standards of the International Conference of Harmonization Guidelines for Good Clinical Practice. The protocol was developed by the investigators and it was approved by the Cyprus National Bioethics Committee and was overseen by an independent safety-monitoring committee evaluating the safety and over-all benefit-risk profiles. The adherence of care providers with the protocol was assessed by an external committee assigned by the funder of the project through reviews of case report forms. All patients gave written informed consent at the time of enrolment.

Randomization and masking

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

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

the conduct of the study were blinded throughout the study. Treating/examining physician, other investigators, pharmacist, neuroradiologist and patients were masked to treatment allocation.

Procedures and end points

The specific omega-3 (re-esterified glycerides) and omega-6 (glycerides) raw materials were purchased according to the required interventions' PUFA-fraction specification (molecular structure, quantity/ratio and quality) with vitamin E (alpha-tocopherol) used as antioxidant stabilizer by the supplier. The vitamins and masking aroma were purchased separately. The mixing of fractions to the final required intervention-composition specification was always performed by the same team of scientists under the supervision of the involved medical biochemist and lipidology specialist, under appropriate conditions every six months. Interventions were stored refrigerated in dark until use. See Table 1 and Supplementary Information Methods 1 and 2 for intervention specification detailed description and study/intervention rational.

Participants were randomly assigned to receive a daily dose of a mixture of 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 A, group A); or composed mixture of pure γ-tocopherol (760mg) dispersed in pure virgin olive oil (16,137 mg) as delivery vehicle (intervention C, group C); or a mixture of intervention formula A with intervention C without the pure virgin olive oil (intervention B, named PLP10, group B); or placebo composed of pure virgin olive oil (16,930mg) (intervention D, group D) (Table 1). 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. The institution's pharmacist was responsible for the appropriate storage and handling of the interventions to the individual participants. The interventions were taken orally once daily 30 minutes before dinner by a dosage calibrated cup for 30 months. The ingredients, ratio and dose have been selected based on their biophysical interrelation to the total known multiple MS causing factors, their biochemical importance and the role expected to play in the normalisation and treatment of the involved complex network of events in the disease pathophysiology. Moreover, the high intake dosage was used to overcome any abnormal dietary accumulation of related agents as a result of patients' food intake habits, irrespective of geographical origin, in relation to the daily consumption ratio of the total fatty acid intake; in order to end-up with omega-3 to omega-6 PUFA indicated physiological body ratio composition of 1:1 wt/wt.

The period beginning from July 1st 2007 (enrolment) until December 31st 2007 (entry baseline) was used for normalization period. This six-month normalization period would allow the interventions' agents to exert their beneficial effect (for the incorporation/normalization of cell membranes by oral PUFA, since they need four to six months to exert pivotal action on immune and neural cells, correction of antioxidant deficiency and body PUFA, and optimal normalization of EPA and DHA levels/ratio).[41-43] The study was completed on December 31st 2009 and the recording of relapses continued until December 31st 2010. More clearly the study included the "normalization period" (July 1st 2007 to Dec 31st 2007), the "on treatment" period (Jan 1st 2008 to Dec 31st 2009) and the 12-month "extended period" (Jan 1st 2010– Dec 31st 2010).

Depending on their clinical status and in accordance with the ethical issues governing clinical trials participants continued receiving the indicative regular available treatments, according to

international guidelines with persistent evaluation of any side-effects and adverse events. The study was designed to end 30 months after enrolment and clinical assessments were scheduled at entry baseline, 3, 9, 15, 21 and 24 months on-treatment. Patients were also clinically examined by the treating neurologist within 48 hours after the onset of new or recurrent neurologic symptoms.

The primary end point was the ARR at two years. A relapse was defined as new or recurrent neurologic symptoms not associated with fever or infection that lasted for at least 24 hours and accompanied by new neurologic signs. Relapses were treated with methyl-prednisolone at a dose of 1g intravenous per day, for three days followed by prednisone orally at a dose of 1mg/kg of weight per day on a tapering scheme for three weeks. The secondary end point at two years was the time to confirmed disability progression, defined as an increase of 1.0 or more on EDSS, confirmed after six months (progression could not be confirmed during a relapse). The final EDSS score was confirmed six months after the end of the study. A post-hoc analysis was performed assessing the proportion of patients free from new or enlarging T2 lesions on brain MRI scans at the end of the study for the per-protocol participants of the group receiving the highest effective intervention versus placebo. Comparison was made only versus the available archival MRI scans up to three months before the enrolment date. MRI scans were performed and blindly analyzed at an MRI evaluation centre. The patients continued to be followed for additional 12 months after completion of the trial and relapses were recorded. Finally, patients were strongly encouraged to remain in the study for follow-up assessments even if they had discontinued the study drug.

Blood samples were collected from all randomized patients at the time of enrolment, at every scheduled clinical assessment and during relapses. To check individual compliance with

intake, the fatty acids composition of patients' red blood cells' membranes was determined, by gas chromatography, according to a standard protocol. The fatty acid analyses were performed after study termination and thus did not influence the blinding.

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

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

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

Statistical analysis

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

Baseline characteristics were compared across all intervention groups by ANOVA or Kruskal-Wallis rank test for continuous variables and by mean Fisher's exact test for categorical variables, as appropriate.

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

For the secondary end-point outcome, the time to disability progression, Kaplan–Meier curves were constructed. Progression to disability and time thereof was compared in a pair-wise fashion for the active interventions versus placebo by the log-rank test in the main analysis and by Cox proportional-hazards models with adjustment for baseline EDSS score, age and DMT in the supportive analysis. Each test was performed with a significance level of 0.05. Multivariate models considered all variables with $P < 0.1$ on univariate models. There was no overt violation of the proportionality assumption.

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

Role of the funding source

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

Results

Study population

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

Among the 80 patients, 20 patients were randomly assigned to each of the three groups to receive the interventions and 20 to receive placebo (Fig 2). Baseline characteristics of both the ITT and the per-protocol populations were similar across groups (Table 2A and 2B). Total drop-out patients completed follow-up until study completion and were included in the ITT analyses (Table 4). Five patients were totally lost to follow before their first scheduled visit and two patients dropped-out before their first scheduled visit progressed to secondary progressive MS. Fifteen patients dropped-out without successfully completing the "normalization" period including five pregnancies. Another 17 patients dropped-out early after entry baseline. Seven patients that dropped out were given monoclonal antibody treatment (natalizumab). Overall, a total of 41 (51%) patients completed the 42-month study (July 2007 through December 31st 2010, including the 12-month extended period) where one patient from group A and two from the placebo group transferred on natalizumab, and 39 (49%) patients either withdrew (drop-out) or lost to follow. Reasons for study-interventions discontinuation are listed in Figure 2.

Efficacy

Relapses

As a proof-of-concept trial we primarily needed to answer whether the interventions were effective for those MS patients who adhere to the assigned treatment, the per-protocol analysis.[45] For the sake of methodological comprehensiveness we also present the ITT analysis as a secondary analysis, to answer a different question, complementary to our core hypothesis; like what happened to MS patients who were placed on the interventions (the effect of assignment).[45] Otherwise, as a result of a high drop-out rate, an ITT analysis will not likely be able to show the superiority of an intervention even if it is effective.[45] In any instance, the proper approach of evaluating a study data is to understand what question

prompted the research and assure that the analysis is appropriate for providing the answer whatever it is called. Both analyses can be performed for a study, using the results from the different analyses to answer different research questions.[45] These interventions are original, composed by a different treatment rational, the SM, never tested before and the important main concern was to evaluate their efficacy and safety based on the per-protocol treated MS patients, without any peripheral noise. The question that had to be answered was: "what happens to the patients that are placed and stick on the specific treatment".

Regarding the per-protocol analysis, during the first year of treatment, the ARR was 0.80, 0.40, 0.78 and 0.83 for the four intervention groups respectively. During the second year, the ARR was 0.90, 0.40, 0.67 and 1.25 for the four intervention groups respectively. Overall, for the two-year primary end point, 8 relapses were recorded for the 10 patients in PLP10 group (0.40 ARR) versus 25 relapses for the 12 patients in placebo (1.04 ARR), a 64% adjusted relative rate reduction (RRR) for the PLP10 group (RRR 0.36, 95% confidence interval (CI) 0.15 to 0.87, p=0.024) (Tables 3A, 5 and Fig 3A and 3C). Excluding 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, Tables 3B and 5). Pair-wise comparisons for the other two groups against placebo did not yield statistically significant results (Tables 3A, 3B). The proportion of patients with ≤1 relapse for the two years on-study was higher in the PLP10 group than in the placebo group (90% versus 42%, p=0.030, Table 5). Seeking to investigate further the observed difference, we compared the relapse rate during the 24 months before entry to the study to the 24 months on-treatment for each intervention group. We observed a statistically significant relative reduction in the ARR (70%) only in the PLP10 group (RRR 0.30; 95% CI 0.14 to 0.65, p=0.003, Table 3A); within-group comparisons for the three other groups ARR reduction was not significant and

remained not significant when natalizumab treated patients were further excluded from the analysis. The effect of PLP10 through time at different time-windows versus placebo for all-time on-study patients is shown in Figures 3A to 3D. The ARR analysis, within time-windows, was not an assigned endpoint, but it could help in the process of evaluating parallel information as the time needed for a specific treatment intervention activity to be evident, as well as the efficacy profile through time. PLP10 reached maximum effect within a year on-treatment (counting from the entry baseline) and remained stable at an ARR of 0.4, displaying a steadily reduced ARR with long free-relapse time-windows. These group B characteristics are considered important parameters of a successful MS treatment where the rule than the exception is the heterogeneity among patients' disease evolution. Specifically, Figure 3D demonstrates the dispersion of relapses throughout the 2-year period of all-time on-study (excluding patients on natalizumab) of PLP10 (n=10) versus placebo (n=10). Placebo, in line with the existing knowledge of how relapse history works in relation to future relapses on MS patients (contagion phenomenon) indicates the expected linearly increased trend of the relapse incidence.[46] The same phenomenon was true for the groups A and C. Finally, during the 12 month post-study extended period (January 1st 2010 to December 31st 2010) all-time on-study patients that received PLP10, showed persistent benefit in the ARR compared to placebo (six relapses for the 10 subjects within PLP10, 0.6 ARR versus19 for the 12 subjects within placebo group, 1.58 ARR) indicating a statistically significant 62% adjusted relative rate reduction in the ARR for the PLP10 group (RRR 0.38, 95% CI 0.12 to 0.99, p=0.046).

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

patients within the placebo arm population were on natalizumab, including the two patients that transferred while all-time on-study versus none within PLP10 group (Supplementary 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). 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 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 patients on natalizumab, there was an increased statistically significant difference between the PLP10 and the placebo group for the same analysis (p=0.006) (Fig 4A). At two years, the cumulative probability of progression was 10% in the PLP10 group and 70% in the placebo group, which represents a decrease of 60 percentage points or a relative 86% decrease in the risk of sustained progression of disability within 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 difference was observed for any comparison of the other two groups compared to placebo (Fig 4A and Supplementary Information Fig 2).

Regarding the ITT analysis, at two years, the cumulative probability of progression was 10% in the PLP10 group and 35% in the placebo group (p=0.052, a trend for an effect), which represents a decrease of 25 percentage points or a relative 71% decrease of the PLP10 for the risk of sustained progression of disability (adjusted hazard ratio 0.22, 95% CI 0.04 to 1.07, p=0.06) (Fig 4B). Two versus seven out of the total randomized patients progressed to confirmed disability in the PLP10 and the placebo groups respectively. No significant differences were observed for groups A or C against placebo (Fig 4B). The mean change in Expanded Disability Status Scale (EDSS) score as a function of visit number is shown in Figure 5.

MRI

Over two years, the MRI results support the overall conclusion from the study that PLP10 has a positive effect on disease activity since only 29% from the PLP10 group as opposed to 67% from the placebo group developed new or enlarging T2 lesions (57% relative risk reduction). Excluding patients on natalizumab there is an increased relative risk reduction (64%) between PLP10 as opposed to placebo with 29% of patients on PLP10 and 80% on placebo with development of new or enlarging T2 lesions (Table 5).

Safety

Over the course of the 30 month study no significant adverse events were reported from any group. According to a questioner procedure the only aetiology for drop-outs was the palatability and smell of the formula preparations. Nausea was reported by two patients. No

abnormal values observed on any of the biochemical and haematological blood tests. No allergic reactions reported.

Discussion

In this proof-of-concept clinical trial assessing the safety and efficacy of three cocktail intervention formulas in RRMS, we observed a significant benefit for the novel PLP10 intervention compared to placebo for both the ARR and the progression to disability. Our results include analyses pertaining to a total of 42 months study collected data, including the 12-month, free of intervention treatment, extension period. We focused on the per-protocol data analysis since it is the appropriate method to best provide the answer to the proof-of-concept trial-addressed question. The high drop-out rate was solely the result of formulas palatability, a common phenomenon in trials using oily interventions where a lot of patients tend to drop-out soon after first dosage. We thus present our main per-protocol analysis, as well as a subgroup analysis excluding patients on natalizumab. We have found a statistically significant reduction in the ARR and the disability progression comparing not only patients on PLP10 versus placebo but also comparing the ARR of the PLP10 patients in the 24-month period prior to the study to the ARR of the 24 months on-study; the observed differences became larger when patients that received natalizumab (the most potent disease modifier) were excluded. The ARR decreased within a year on PLP10 and significantly remained stable until study completion. Statistically significant difference of ARR between patients on PLP10 versus placebo continued for the additional 12 month extended period (persistent effect) without significant difference on DMT. These clinical findings are supported by the results regarding the MRI analysis where the proportion of patients free from new or enlarging brain T2 lesions was also higher in PLP10 group versus placebo. The persistent effect within the extended period it is considered of major importance and supportive of the results since it is

in agreement with the very long washouts, reported necessary, for omega-3 fatty acids and especially DHA to return towards pretreatment values within the fatty acids of plasma, platelets, monocytes and red blood cells.[42] This study also provides important 30-month, placebo-controlled information about the safety of PLP10, A and C interventions, where no any adverse or severe side effects have been reported.

As medications used to treat MS become increasingly highly specific and potent, attention to safety is paramount. Current available treatments are products of reductionism, partially effective, associated with severe side effects without (re)myelinating or neuroprotective abilities. Interferons and glatiramer acetate, the most widely used first-line MS drugs available today, are associated with the least severe side-effects among MS therapies but they are reported with only 29-33% ARR reduction and with no significant effects on the progression of disability. Natalizumab as previously discussed and Fingolimod with 54% ARR reduction (without significant benefit on the progression of disability) are second-line drugs associated with severe side-effects.[47, 48]

No existing MS treatment has ever been designed as a result of SM concept approach or with a potential to effectively stimulate intrinsic remyelinating and neuroprotecting mechanisms or exert such an action. Now we propose that a holistic SM model approach has to be applied by synchronized action on all involved perturbed mechanisms. PLP10 has innovative characteristics like no any other intervention or medication tried before for MS treatment, with unique efficacy abilities through different mechanisms of action, probably by the synergistic effect of its constituent ingredients. PLP10 has all the characteristics of a medical food with the action to feed a normal metabolic process by supplying nutritional structural membrane precursors, building blocks, and vitamins from dietary sources that enhance

remyelination and neuroprotection and simultaneously promote normalization of all cellular membranes lipid content. The intention is to normalize the specific nutritional requirements of the MS patients.

Different factors and molecular entities appear to be part of the possible aetiology for MS with specific PUFA and antioxidants found to be key substances related to all known pathogenic and recovery mechanisms. But, it is well established that MS patients are characterized with significant deficiencies in antioxidant efficiency as well as in PUFA levels in blood and cellular membranes.[11, 49-51]

According to one hypothesis, the change in the ratio of omega-3 to omega-6, due to increasing consumption of omega-6 PUFA, meaning high accumulation of AA, in the Western diet, may be one of the major factors responsible for the increasing incidence of inflammatory diseases relative to populations. In the Western diet, the ratio of omega-3 to omega-6 is about 1:20–30; in populations that consume fish-based diets, the ratio is about 1:1–2.[52, 53] The intervention daily dose was aiming and believed to be high enough to restore/amplify body efficient antioxidant activity and ensure cellular membranes lipid profile normalization (PUFA content) and simultaneously potentiate involvement of the ingredients in the anti-inflammatory and recovery mechanisms. Diet fatty acid molecules need about six months period to exert their beneficial effect and this essential parameter was for the first time under consideration in our study design (normalization period).[42] This chronotherapy parameter it is of major importance in line with the SM treatment philosophy and if it is not included in the trial design the possibility of misleading result evaluation greatly increases. In fact, considering that omega-3 supplementation can release and replace excess AA within the

cellular membranes, we can speculate that an increased inflammatory activity can possibly result during the first six months of supplementation (during normalization period).

The maintenance of myelin requires continued turnover of its components throughout life.[54,55] In addition to the EPA, DHA, LA, and GLA, PLP10 was containing limited quantities of other structural PUFA, specific MUFA (mostly oleic acid) and SFA (palmitic and stearic acid), specifically to provide a direct source for neuronal cell membranes rehabilitation and for (re)myelination and neuroprotection since they are all major components, precursors and building blocks of any new physiological myelin and cellular membranes in general. Assembly of the correct molecules into myelin membrane may be especially critical during active synthesis. Possibly, if critical constituents aren't available or are metabolically blocked, amyelination, dysmyelination or demyelination may ensue.[56]

The well known and established safety of the ingredients used and the protocol guidelines were supportive reasons for us to proceed with the clinical study even though with limitation on the pre-estimation of required trial sample size as it was discussed in method section. The adherence of the subjects is another issue but the duration of the study (42 months) is adding power to the results;[44] having the research questions been consciously and carefully approached and answered. Furthermore, the statistical methodologies used along with the appropriate adjustments, broadly accepted for MS clinical trials, power the findings, results, and significance. The baseline characteristics of the treatment arms could possibly be considered indicative of four very active groups of patients but that was the result of the limited number of RRMS population eligible for the study within Cyprus. On the other hand the balanced baseline characteristics without statistical differences, the statistical adjustments (for all important baseline parameters i.e. relapses, EDSS, age and DMT) and the

randomization within four different groups are the safety valves against data misinterpretation. Yet, in small randomized control trials with a high drop-out rate, the per-protocol analysis could be affected by the characteristics of the patients dropping out. In order to safeguard our findings in the best possible way under the circumstances, we proceeded to adjusting for confounders. Moreover, we cannot discard our finding as a false positive, given that this is a randomized, double-blind, placebo-controlled clinical trial and, despite its small sample size, represents a piece of evidence that only a larger randomized controlled trial can replicate or refute. It is possible to question why DMTs efficacy cannot be emerged out of the data analysis, of the four treatment arms, and in accordance to their published values. We believe that the limited efficacy of the DMTs, the sample size and the statistical adjustments were strong limiting determining factors for such an indication to be countable. An additional argument is that the efficacy reported for the analysis of pre-treatment (24 months before entry baseline) versus on-trial ARR could be considered as potentially biased due to differences of how relapses were defined during the course of a study compared to pre-treatment period; or due to regression to the mean or placebo effect. This analysis was performed as an additional exploratory analysis that we were able to do due to the availability of data. The relapses of the two pre-treatment years were drawn out of the patients' archival records by the same treating neurologist involved in the study (MP), and according to the patients' hospitalization date for receiving intravenous methyl-prednisolone. This analysis was not used as a primary or a secondary end-point under investigation although it is usually reported by many clinical studies. As a matter of fact many early phase trials are based only on such an analysis (before versus after treatment results). In almost all MS trials the number of relapses within the two years before baseline is a factor under adjustment for the statistical analyses.[48] The inclusion of the post-hoc MRI analysis is another limiting factor that needs attention since it was used as an additional aside exploratory

approach (due to study budget limitations it was not possible to be used as a formal endpoint); but the MRI evaluation was blinded and can be considered as representative of the randomized subjects within the treatment arms. As far as the regression to the mean and the placebo effect concerns we believe that the 6-month normalization period is an accountable and valuable eliminating factor of the possible effect; as well as the presence of four groups, where only the PLP10 treatment arm is associated with statistically significant efficacy versus placebo. It is a placebo-controlled study after all.

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

It is commonly accepted that nutrition is one of the possible environmental factors involved in the pathogenesis of MS, but its role as complementary MS treatment is unclear and largely disregarded.[57] It is well known that the majority of the patients suffering from MS they do use dietary supplements for a variable length of time and they prefer supplement type of

"help" over conventional drugs.[58] Dietary antioxidants and fatty acids may influence the disease process in MS by reducing immune-mediated inflammation, oxidative stress and excitotoxic damage.[11] Present data reveal that healthy dietary molecules have a pleiotropic role and are able to change cell metabolism from anabolism to catabolism and down-regulate inflammation by interacting with enzymes, nuclear receptors and transcriptional factors.[57] The present study, for the first time provides strong link evidence between dietary, metabolic, immunological, and neurobiological aspects of MS after three quarters of a century of unsuccessful scientific efforts. This might probably be the beginning of opening new horizons and new avenues in the approach of MS prevention and treatment, and possibly of other multifactorial chronic diseases, including neurodegenerative and autoimmune as well.

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.

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.

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

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.

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.

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 1year**	**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 by1 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 by1 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
† 1out 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 for the protocol and study design. M.C.P was the evaluator of patients' clinical progress signs and the treatment physician. I.S.P and G.N.L were involved in the non-pharmacologic treatment-related care. I.S.P performed the literature search and both I.S.P and G.N.L contributed on the intervention formulation and composition rational. I.S.P supervised the composition procedure of the interventions and the fatty acid profile analysis of the red blood cell membranes. E.E.N and I.S.P performed the statistical analyses. E.E.N was an independent scientist. All authors vouch for the accuracy and completeness of the data and the statistical analyses.

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Ethical approval: Cyprus National bioethics committee (reference ΕΕΒΚ/ΕΠ/2005/10).

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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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Figure legends

Figure 1. Omega-6 and omega-3 PUFA, their respective metabolic derivatives and their possible effects on inflammation.

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

Abbreviations: PL, phospholipid; IFN-γ, interferon γ; IL-2, interleukin 2; NFκB, nuclear factor kappa B; PGE2, prostaglandin E2; PPARγ, peroxisome proliferator-activated receptor γ; PUFAs, polyunsaturated fatty acids; TGFβ, 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.

Figure 2. Study Flowchart

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. **

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

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

Panel D demonstrates the dispersion of relapses throughout the 2-year period of all-time on-study (excluding patients on natalizumab) of PLP10 (n=10) *vs.* placebo (n=10). Placebo

shows an irregular dispersion of relapses in relation to PLP10 group with linear increasing trend wile PLP10 shows a stabilized linear trend. By using the per-protocol model where patients on natalizumab were excluded, we could compare the number of relapses on a same number of patients.

** Including the patients on natalizumab.

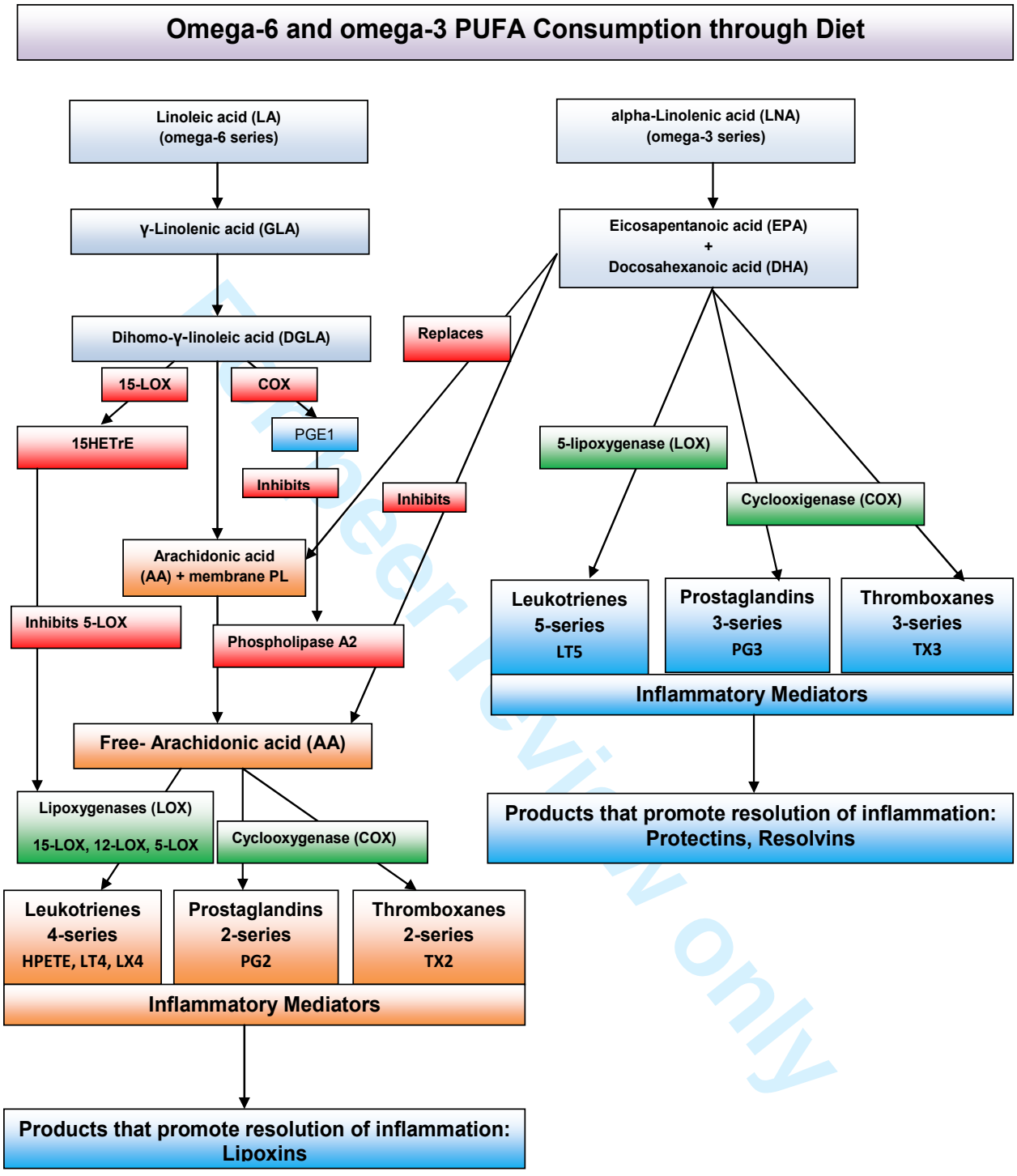
Figure 4. Panel 4A demonstrates the Kaplan–Meier plot of the time to sustained progression of disability among all-time on-study patients, excluding patients on natalizumab, receiving intervention A, PLP10 and C as compared with placebo. PLP10 reduced the risk of sustained progression of disability by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

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

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

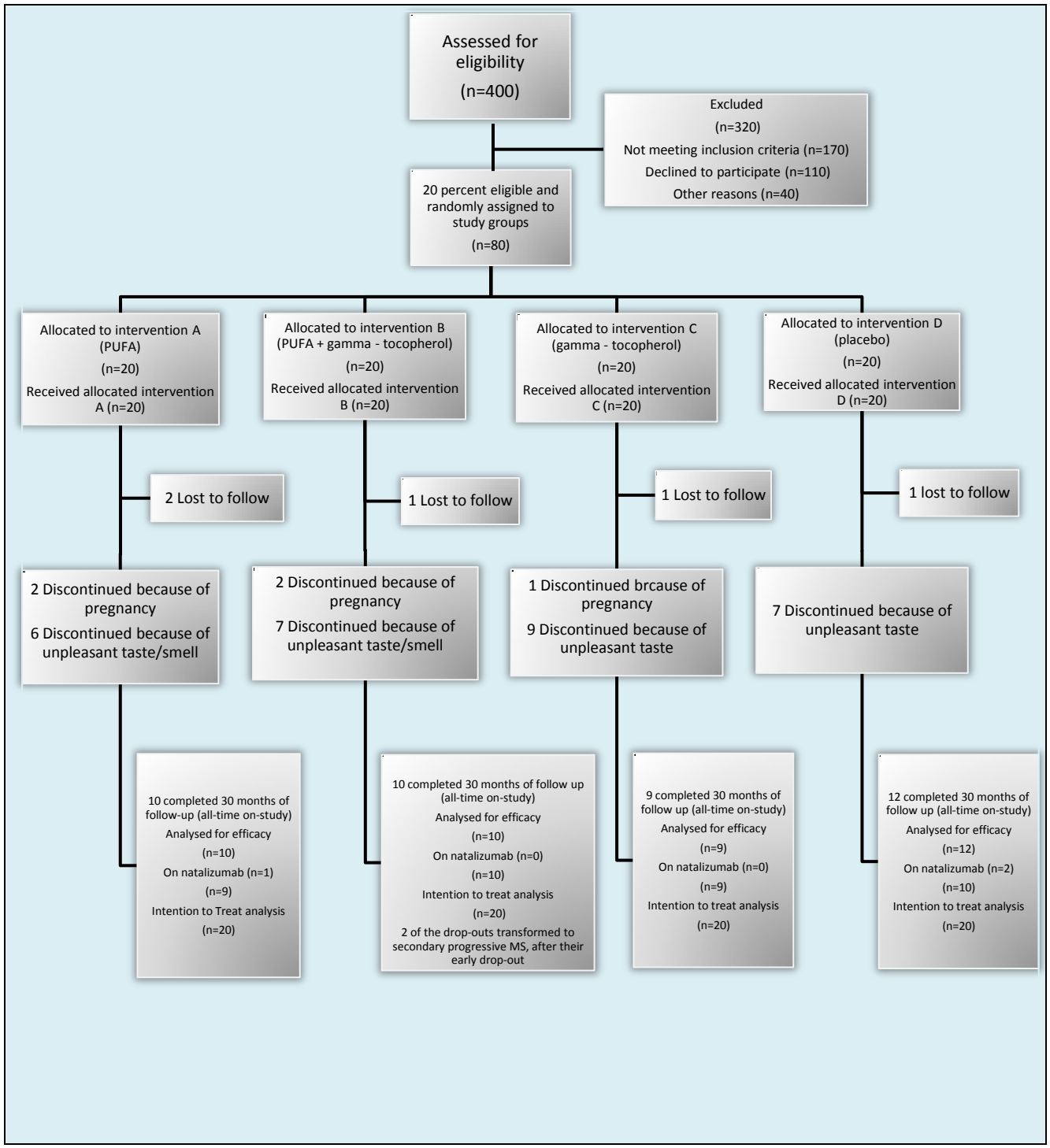
¶ Including patients on natalizumab

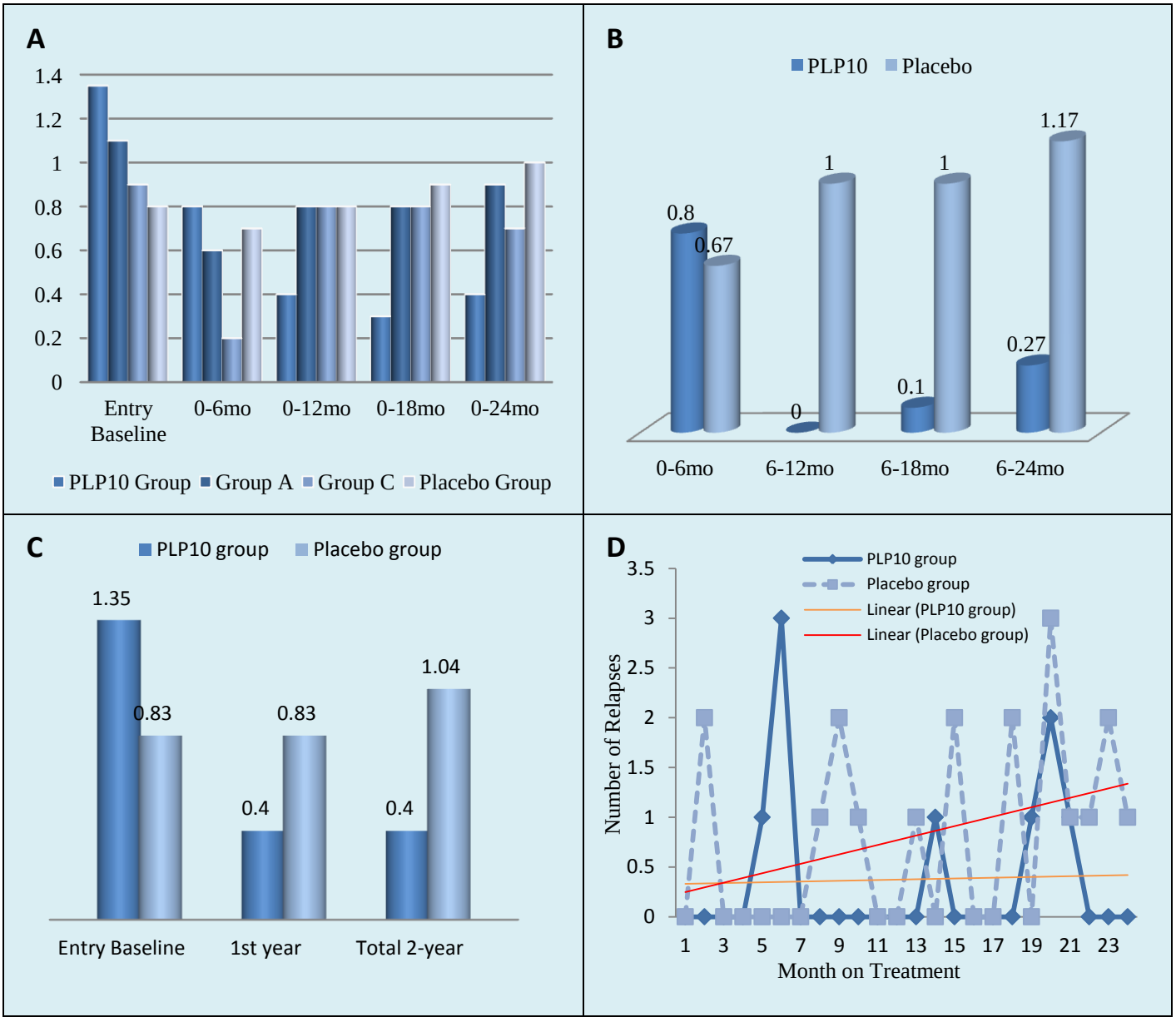
¶¶ Excluding patients on natalizumab

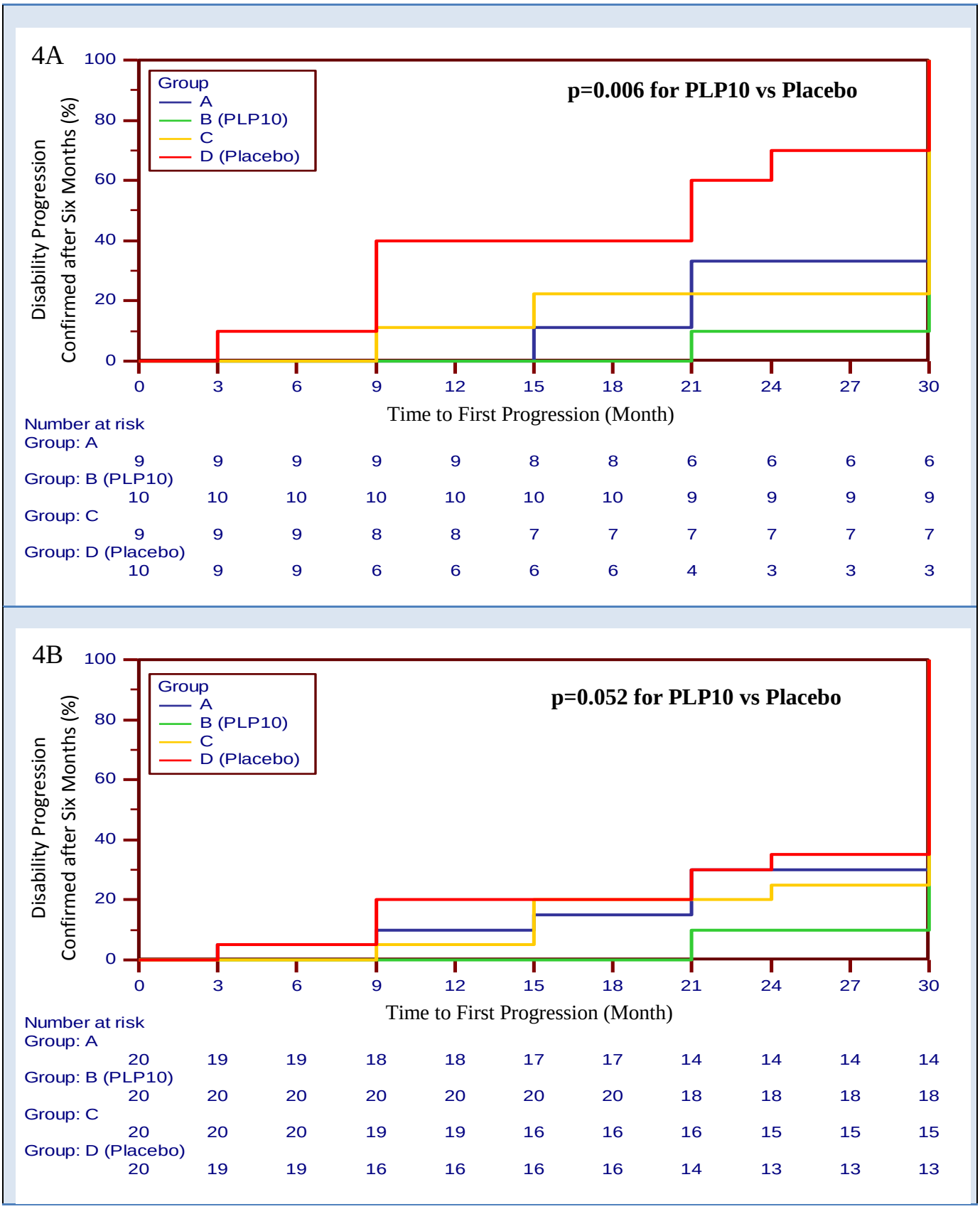


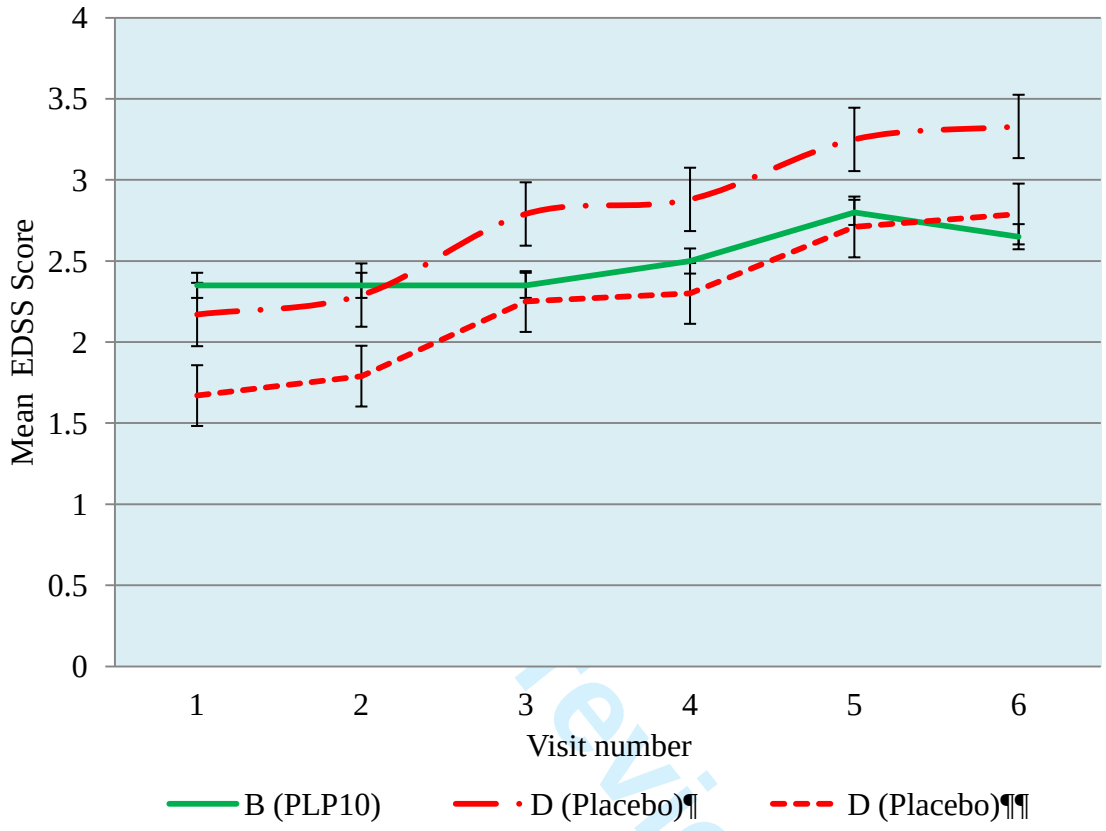
Possible effects on inflammation:

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


Assessed for eligibility
(n=400)
Excluded
(n=320)
Not meeting inclusion criteria (n=170)
Declined to participate (n=110)
Other reasons (n=40)
20 percent eligible and randomly assigned to study groups
(n=80)
Allocated to intervention A (PUFA)
(n=20)
Received allocated intervention A (n=20)
Allocated to intervention B (PUFA + gamma - tocopherol)
(n=20)
Received allocated intervention B (n=20)
Allocated to intervention C (gamma - tocopherol)
(n=20)
Received allocated intervention C (n=20)
Allocated to intervention D (placebo)
(n=20)
Received allocated intervention D (n=20)
2 Lost to follow
1 Lost to follow
1 Lost to follow
1 lost to follow
2 Discontinued because of pregnancy
6 Discontinued because of unpleasant taste/smell
2 Discontinued because of pregnancy
7 Discontinued because of unpleasant taste/smell
1 Discontinued brcause of pregnancy
9 Discontinued because of unpleasant taste
7 Discontinued because of unpleasant taste
10 completed 30 months of follow-up (all-time on-study)
Analysed for efficacy
(n=10)
On natalizumab (n=1)
(n=9)
Intention to Treat analysis
(n=20)
10 completed 30 months of follow up (all-time on-study)
Analysed for efficacy
(n=10)
On natalizumab (n=0)
(n=10)
Intention to treat analysis
(n=20)
2 of the drop-outs transformed to secondary progressive MS, after their early drop-out
9 completed 30 months of follow up (all-time on-study)
Analysed for efficacy
(n=9)
On natalizumab (n=0)
(n=9)
Intention to treat analysis
(n=20)
12 completed 30 months of follow up (all-time on-study)
Analysed for efficacy
(n=12)
On natalizumab (n=2)
(n=10)
Intention to treat analysis
(n=20)


A
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Entry Baseline
0-6mo
0-12mo
0-18mo
0-24mo
PLP10 Group
Group A
Group C
Placebo Group
B
PLP10
Placebo
0.8
0.67
1
0
1
0.1
1.17
0.27
0-6mo
6-12mo
6-18mo
6-24mo
C
PLP10 group
Placebo group
1.35
0.83
0.4
0.83
0.4
1.04
Entry Baseline
1st year
Total 2-year
D
PLP10 group
Placebo group
Linear (PLP10 group)
Linear (Placebo group)
Number of Relapses
3.5
3
2.5
2
1.5
1
0.5
0
1 3 5 7 9 11 13 15 17 19 21 23
Month on Treatment


4A
Group
A
B (PLP10)
C
D (Placebo)
p=0.006 for PLP10 vs Placebo
Disability Progression
Confirmed after Six Months (%)
100
80
60
40
20
0
0 3 6 9 12 15 18 21 24 27 30
Time to First Progression (Month)
Number at risk
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
4B
Group
A
B (PLP10)
C
D (Placebo)
p=0.052 for PLP10 vs Placebo
Disability Progression
Confirmed after Six Months (%)
100
80
60
40
20
0
0 3 6 9 12 15 18 21 24 27 30
Time to First Progression (Month)
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



Supplementary Information

Table of Content

Supplementary Information Methods 1

Intervention and study rational. We are using high dosage of 1:1 omega-3:omega-6 polyunsaturated (PUFA) aiming to overpass and normalize global diet regional traditions and habits in relation to abnormal PUFA/saturated fatty acids (SFA)/monounsaturated fatty acids (MUFA) daily ratio consumption irrespective of the quantities consumed; and enough to equilibrate patients' diet in line with the recommended more physiologic omega-3:omega-6 ratio of about 1:1-4 wt/wt as reported by Simopoulos, 2002. In addition to correct existing deficiencies, cell membrane abnormalities, specifically of the immunopathological system and blood mononuclear peripheral cells, and high enough for availability and immediate ongoing modulation of the involved pathogenic mechanisms and network of events in MS. The high dosage is also required to overpass the quantity limitations, previously discussed, of diet-consumed PUFAs for cellular incorporation, especially in the central nervous system (CNS) of adults. Additionally, fatty acids (FAs) must first cross the intestinal epithelium before reaching the different tissues, where digestion and absorption constitute further problems in their availability (Carlier, H, 1991). Omega-3 PUFA are used in re-esterified form to eliminate unwanted disturbances, at the sides of action, by other fatty acids and molecules present in crude fish oils but also to increase the bioavailability of the FA since triglycerides have been shown to be associated with much higher bioavailability (Dyerberg et al, 2010). Linoleic (LA) and gamma linolenic acid (GLA) are essential structure molecules and important for any physiological (re)generation of cell membrane. GLA quantity is doubled to LA to ensure high direct production of dihomo-gamma-linolenic acid (DGLA), from GLA when LA cannot be metabolized, due to desaturase deficiency or malfunction. Such a reduced capacity to convert LA to GLA has been associated with aging, diabetes, alcoholism, atopic dermatitis, premenstrual syndrome, rheumatoid arthritis, cancer and cardiovascular diseases (Bolton-Smith et al, 1997; Horrobin, 1990; Leventhal et al, 1993). This is going to result in the increase of DGLA relative to arachidonic acid (AA) with DGLA promoting production of prostaglandin (PG)E1 but also inhibition of phospholipase (PL)A2: two major reasons and rational for their use. If other metabolic problems are involved within the omega-6 series and the normal metabolites are not produced then the eicosapentaenoic acid EPA available in PLP10 will substitute the function of DGLA, as a competitive inhibitor of AA for PLA2. In both cases the pro-inflamatory leucotrienes, prostaglandines of the 2-series (PG2) and thromboxanes of the 2 series including the platelet-activator factor (PAF) will be attenuated. The synthesis of AA from DGLA by Δ5 desaturase promoted by LA/GLA supplementation is very limited in humans as a result of limited activity of the enzyme (Yang-Yi & Robert, 1998). AA in the body is mostly available through diet. EPA and docosahexaenoic acid (DHA) are both physiologically important and crucial structured molecules able to substitute excess AA and SFA within the cell membranes. EPA will contribute to the inhibition (competitive to AA) of PLA2, joining the co-supplied omega-6 PUFA but will also participate in the production of anti-inflammatory leukotrienes, prostaglandins of the 3-series (PG3) and thromboxane (TX3) along with DHA, both found in the PLP10 intervention. Moreover EPA will replace AA of the membrane phospholipids and both omega-3 PUFA will contribute replacing abnormal quantities of SFA and excess AA. DHA is used in 3:1 ratio to EPA to cover any possible inabilities of EPA to be metabolized, high enough to strongly promote high production of the aforementioned anti-inflammatory

eicosanoids and cytokines and to be incorporated into CNS cell membranes where DHA should be the major PUFA present, replacing other FA, probably saturated and excess of AA. EPA, DHA, LA and GLA along with the rest of the other ingredients used ("other" omega-3 PUFA, SFA and MUFA that are usually found in the cell membranes of healthy people in limited quantities) in the intervention regimen are for their availability as minor structural constituents of physiological cellular membranes integrity, fluidity and overall function as building blocks for myelin repair and/or myelination. Furthermore the PUFA used within the cocktail intervention aimed to manipulate all other pathophysiological pathways that are reported to be able to: as previously discussed including gene transcription for neuroprotection and remyelination. Furthermore, PUFA are used to ensure the integrity of blood brain barrier (BBB) and to modulate the gelatinases responsible for the T cell migration within the CNS. Three different antioxidant vitamins (vitamin E, mostly as alpha-tocopherol, gamma (γ)-tocopherol (vitamin E isoform) and vitamin A) are used in the regimen preparation to support the cellular antioxidant defenses but also to protect peroxidation of the supplied increased amounts of PUFA. Alpha-tocopherol low-molecular-weight antioxidants will contribute to radical scavenging, interfering with gene transcription, protein expression, enzyme activity and metal chelation (van Meeteren et al, 2005). Vitamin E (alpha tocopherol) and vitamin A are used as antioxidants for the protection of the excess supplemented PUFA, with alpha-tocopherol been demonstrated to protect against peroxynitrite-induced oxidative damage, as well as able to efficiently detoxify hydroxyl, perhydroxyl and superoxide free radicals (the elevated reactive oxygen species (ROS)); each one with different mechanism of action, increasing the effect capability (Vatassery et al, 1998b; van Meeteren, 2005). Gamma-tocopherol is used in high dosage since its half life is very short compared to alpha-tocopherol and has been demonstrated to specifically protect against nitro-radicals. Tocopherols can also exert non-antioxidant properties, including modulation of cell signaling and immune function, regulation of transcription, and induction of apoptosis as previously discussed (van Meeteren et al, 2005).

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

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

adjunct to existing pharmaceuticals produced by reductionism, can be proven therapeutically superior to any available treatment for MS.

Bolton-Smith C, Woodward M, Tavendale R (1997) Evidence for age-related differences in the fatty acid composition of human adipose tissue, independent of diet. *European Journal of Clinical Nutrition* **51:** 619-624

Carlier H, Bernard A, Caselli C (1991) Digestion and absorption of polyunsaturated fatty acids. *Reproduction, nutrition, development* **31:** 475-500

Dyerberg J, Madsen P, Møller JM, Aardestrup I, Schmidt EB (2010) Bioavailability of marine n-3 fatty acid formulations. *Prostaglandins, leukotrienes, and essential fatty acids* **83:** 137-141

Horrobin DF (1990) Gamma-linolenic acid. *Rev Contemp Physiol* **1:** 1-41

Leventhal LJ, Boyce EG, Zurier RB (1993) Treatment of rheumatoid arthritis with gammalinolenic acid. *Annals of Internal Medicine* **119:** 867-873

Simopoulos AP (2002) The importance of the ratio of omega-6/omega-3 essential fatty acids. *Biomedecine & Pharmacotherapy* **56:** 365-379

van Meeteren ME, Teunissen CE, Dijkstra CD, van Tol EAF (2005) Antioxidants and polyunsaturated fatty acids in multiple sclerosis. *Eur J Clin Nutr* **59:** 1347-1361

Yang-Yi F, Robert SC (1998) Importance of Dietary g-Linolenic acid in human health and nutrition. *The Journal of Nutrition* **128:** 1411-1414

Vatassery GT, Smith WE, Quach HT (1998b) Alpha-tocopherol in rat brain subcellular fractions is oxidized rapidly during incubations with low concentrations of peroxynitrite. *Journal of Nutrition*128:152–157.

Supplementary Information Methods 2

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

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

The daily intervention formula agent dosages were:

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

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

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

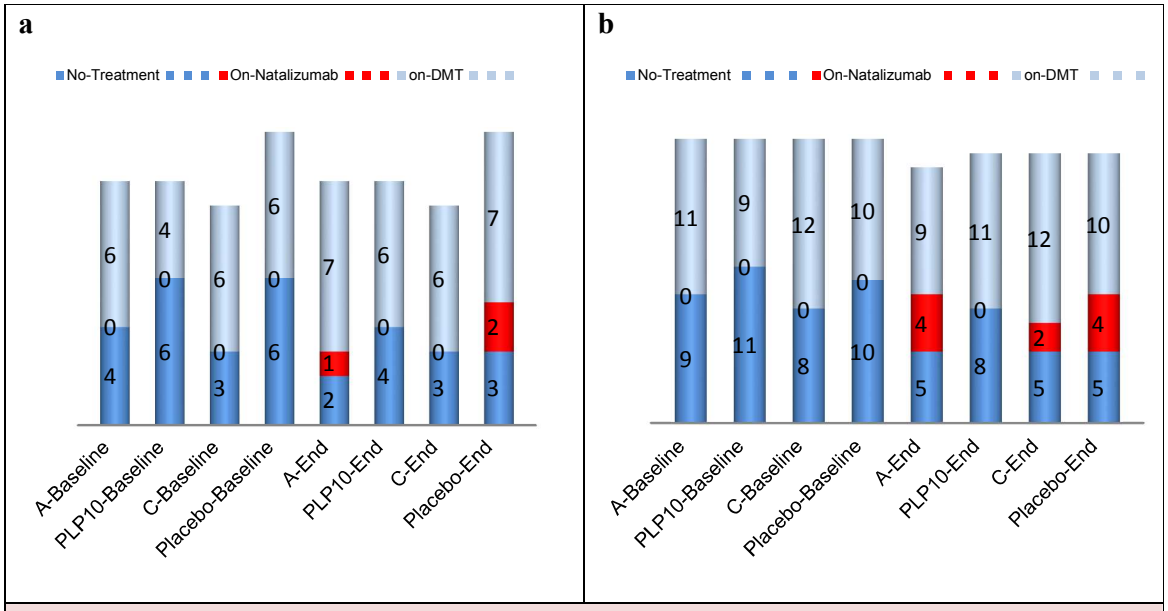
Intervention formula D daily dosage**:** pure virgin olive oil (16930mg).

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

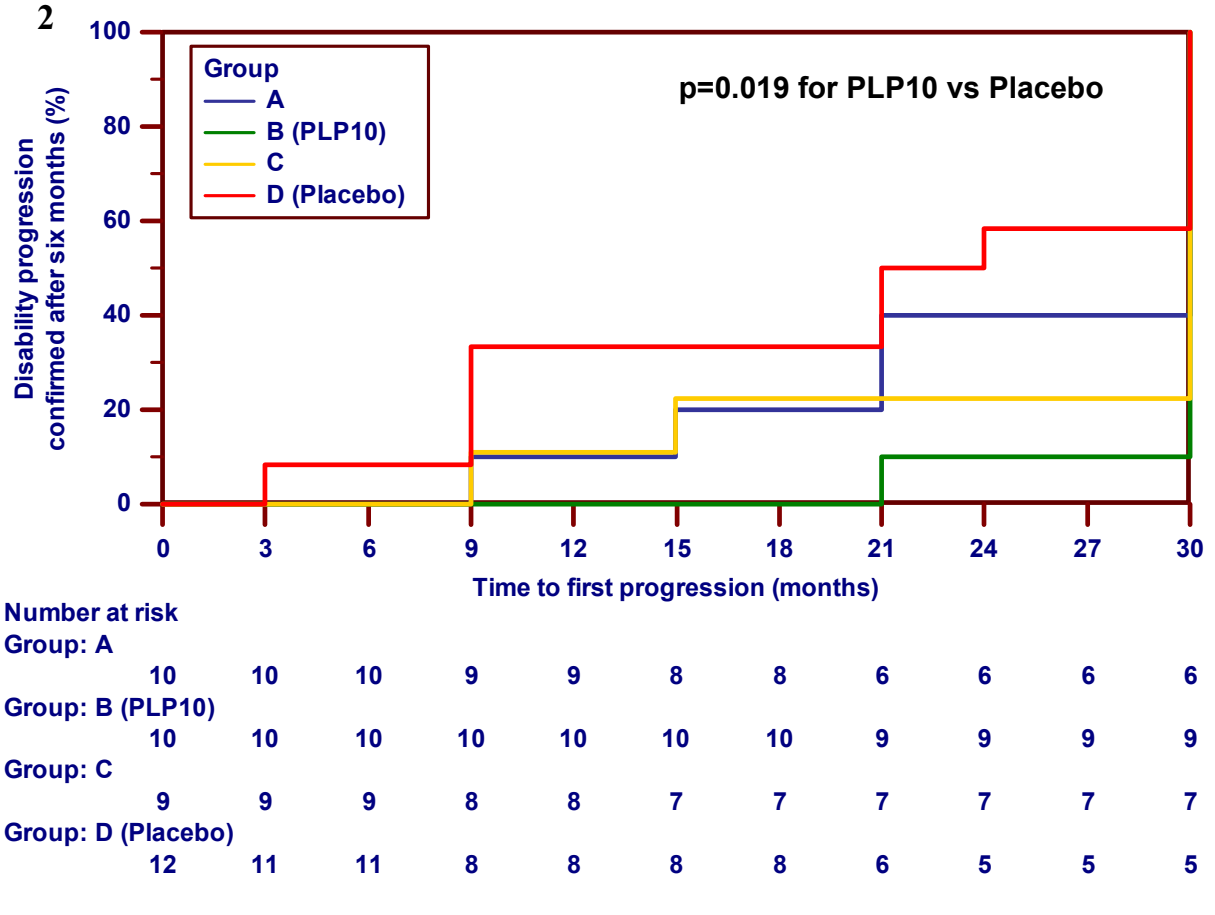
The specific omega-3 related fraction, according to specifications required for the interventions was prepared and purchased from EPAX AS, Aalesund, Norway; as re-esterified glycerides from fish body oils as a source. The specific omega-6 PUFA, MUFA and SFA related fraction, according to required specifications,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

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

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



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.



Supplementary Information Figure 2 | Kaplan–Meier estimates for the time to disability progression. Kaplan–Meier plot of the time to sustained progression of disability among all-time on-study patients, including patients on natalizumab, receiving intervention A, PLP10 and C *vs.* placebo. Intervention PLP10 reduced the risk of sustained progression of disability by 83% over two years (p=0.019). The cumulative probability of progression was 10% in the intervention B group and 58% in the placebo group. Intervention formula A reduced the risk of sustained progression of disability by 32% (p=0.301) and intervention formula C by 62% (p=0.109).

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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.

BMJ open

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:	*BMJ Open*
Manuscript ID:	bmjopen-2012-002170.R2
Article Type:	Research
Date Submitted by the Author:	04-Feb-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
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A novel oral nutraceutical formula of omega-3 and omega-6 fatty acids with vitamins (PLP10) in relapsing remitting multiple sclerosis: a randomized, double-blind, placebo-controlled proof-of-concept clinical trial

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Abstract

Objective To assess whether our three novel interventions, formulated based on systems 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.

Results A total of 41 (51%) patients completed the 42-month trial. Overall, 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). No adverse events were reported. Interventions A (10 patients) and C (9 patients) showed no significant efficacy.

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

Trial registration International Standard Randomized Controlled Trial, number ISRCTN87818535.

Introduction

Multiple sclerosis (MS) is a complex multifactorial disease that results from interplay between as yet unidentified environmental factors and susceptibility genes.[1-3] Together, these factors trigger a cascade of events, involving engagement of the immune system, inflammatory injury of myelin, axons and glia, functional recovery and structural repair, gliosis, and neurodegeneration.[4] The bio-mechanisms involved are: immune-mediated inflammation, oxidative stress and excitotoxicity.[5-9] These mechanisms may all contribute to oligodendrocyte and neuronal damage and even cell death, hence promoting disease progression. The increasing prevalence of 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.

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

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

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

In vitro and in vivo studies have demonstrated that dietary EPA, DHA, LA, and GLA can be implicated and modulate almost all known complex network of events and pathways repertoire in MS pathophysiology. Brain membrane fatty acid composition can be modified with dietary supplementation, but the process has been showed to be age dependent (it takes much longer in adults versus developing brains) as well as possibly dependent on the quantities of the dietary/supplemented PUFAs.[14] Both human and animal studies proved that diets high in DHA and EPA increase the proportion of these PUFA in the membranes of inflammatory cells and reduce the levels of AA.[12, 15] The anti-inflammatory properties of

omega-3 include production of PGs and TXs of the 3-series and LTs of the 5-series (Fig 2).[14, 16] Resolvins and protectins are biosynthesized from omega-3 fatty acids via cyclooxygenase-2/lipoxygenase (COX-2/LOX) pathways and they promote control of inflammation in neural tissues (Fig 2).[17-21] T-cell proliferation in acute and chronic inflammation can be reduced by supplementation with either omega-6 or omega-3 PUFA.[22] Furthermore, vitamin E is an important antioxidant that can interrupt the propagation of free radical chain reactions.[23] Vitamin E (alpha-tocopherol, an isoform of vitamin E) efficiently detoxifies hydroxyl, perhydroxyl and superoxide free radicals.[24] However γ-tocopherol (another isoform of vitamin E) seems to be more efficiently implicated in trapping NO radicals.[25] In addition alpha–tocopherol exerts non-antioxidant properties, including modulation of cell signaling and immune function, regulation of transcription, and induction of apoptosis.[26]

Moreover, omega-3 fatty acids electrophilic derivatives formed by COX-2, in activated macrophages can stimulate the nuclear respiratory factor (Nrf2) inducing the transcription of neuroprotective and antioxidant related genes, and can activate the peroxisome proliferator-activated receptor (PPAR)γ for anti-inflammatory response.[27-29] In animal studies, EPA and DHA proved to be endogenous ligands of RXRs, with positive effect on neurogenesis.[30] Additionaly, in 2008 Salvati and coworkers reported evidense of accelerated myelination in DHA- and EPA-treated animals.[32] Moreover, DHA and EPA are reported to significantly decrease the levels of metalloproteinases (MMP) -2, -3, -9, and -13 with a significant role in the migration of lymphocytes into the CNS by inducing disruption of the blood brain barrier (BBB), an important step in the formation of MS lesions.[33-39]

Based on the above, specific PUFA and antioxidant vitamins fulfill the criterion of biologic plausibility and have the potential to diminish MS symptoms severity and activity, even

promoting recovery (remyelination).[11] Overall, PLP10 contains multiple ingredients (omega-3, omega-6 and other fatty acids and vitamins) potentially able to modulate key interconnected components (i.e. genes, proteins) and structural molecules (i.e. cellular membrane lipids, receptors) within the functional network of events of MS pathogenesis.[40]

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

Methods

Patients

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

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

The study was conducted in accordance with the standards of the International Conference of Harmonization Guidelines for Good Clinical Practice. The protocol was developed by the investigators and it was approved by the Cyprus National Bioethics Committee and was overseen by an independent safety-monitoring committee evaluating the safety and over-all benefit-risk profiles. The adherence of care providers with the protocol was assessed by an external committee assigned by the funder of the project through reviews of case report forms. All patients gave written informed consent at the time of enrolment.

Randomization and masking

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

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

the conduct of the study were blinded throughout the study. Treating/examining physician, other investigators, pharmacist, neuroradiologist and patients were masked to treatment allocation.

Procedures and end points

The specific omega-3 (re-esterified glycerides) and omega-6 (glycerides) raw materials were purchased according to the required interventions' PUFA-fraction specification (molecular structure, quantity/ratio and quality) with vitamin E (alpha-tocopherol) used as antioxidant stabilizer by the supplier. The vitamins and masking aroma were purchased separately. The mixing of fractions to the final required intervention-composition specification was always performed by the same team of scientists under the supervision of the involved medical biochemist and lipidology specialist, under appropriate conditions every six months. Interventions were stored refrigerated in dark until use. See Table 1 and Supplementary Information Methods 1 and 2 for intervention specification detailed description and study/intervention rational.

Participants were randomly assigned to receive: in group A, a daily dose of a 19.5ml mixture of 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 A); in group B PLP10, a daily dose of a 19.5ml mixture of 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 pure γ-tocopherol (760mg) plus citrus-aroma (intervention B); in group C, a daily dose of a 19.5ml mixture of pure γ-tocopherol (760mg) dispersed in pure virgin olive oil (16,137 mg) plus citrus-aroma

(intervention C) and in group D placebo, a daily dose of a 19.5ml mixture of pure virgin olive oil (16,930mg) plus citrus-aroma (intervention D) (Table 1). The institution's pharmacist was responsible for the appropriate storage and handling of the interventions to the individual participants. The interventions were taken orally once daily 30 minutes before dinner by a dosage calibrated cup for 30 months. The ingredients, ratio and dose have been selected based on their biophysical interrelation to the total known multiple MS causing factors, their biochemical importance and the role expected to play in the normalisation and treatment of the involved complex network of events in the disease pathophysiology. Moreover, the high intake dosage was used to overcome any abnormal dietary accumulation of related agents as a result of patients' food intake habits, irrespective of geographical origin, in relation to the daily consumption ratio of the total fatty acid intake; in order to end-up with omega-3 to omega-6 PUFA indicated physiological body ratio composition of 1:1 wt/wt.

The period beginning from July 1st 2007 (enrolment) until December 31st 2007 (entry baseline) was used for normalization period. This six-month normalization period would allow the interventions' agents to exert their beneficial effect (for the incorporation/normalization of cell membranes by oral PUFA, since they need four to six months to exert pivotal action on immune and neural cells, correction of antioxidant deficiency and body PUFA, and optimal normalization of EPA and DHA levels/ratio).[41-43] The study was completed on December 31st 2009 and the recording of relapses continued until December 31st 2010. More clearly the study included the "normalization period" (July 1st 2007 to Dec 31st 2007), the "on treatment" period (Jan 1st 2008 to Dec 31st 2009) and the 12-month "extended period" (Jan 1st 2010– Dec 31st 2010).

Depending on their clinical status and in accordance with the ethical issues governing clinical trials participants continued receiving the indicative regular available treatments, according to international guidelines with persistent evaluation of any side-effects and adverse events. The study was designed to end 30 months after enrolment and clinical assessments were scheduled at entry baseline, 3, 9, 15, 21 and 24 months on-treatment. Patients were also clinically examined by the treating neurologist within 48 hours after the onset of new or recurrent neurologic symptoms.

The primary end point was the ARR at two years. A relapse was defined as new or recurrent neurologic symptoms not associated with fever or infection that lasted for at least 24 hours and accompanied by new neurologic signs. Relapses were treated with methyl-prednisolone at a dose of 1g intravenous per day, for three days followed by prednisone orally at a dose of 1mg/kg of weight per day on a tapering scheme for three weeks. The secondary end point at two years was the time to confirmed disability progression, defined as an increase of 1.0 or more on EDSS, confirmed after six months (progression could not be confirmed during a relapse). The final EDSS score was confirmed six months after the end of the study. A post-hoc analysis was performed assessing the proportion of patients free from new or enlarging T2 lesions on brain MRI scans at the end of the study for the per-protocol participants of the group receiving the highest effective intervention versus placebo. Comparison was made only versus the available archival MRI scans up to three months before the enrolment date. MRI scans were performed and blindly analyzed at an MRI evaluation centre. The patients continued to be followed for additional 12 months after completion of the trial and relapses were recorded. Finally, patients were strongly encouraged to remain in the study for follow-up assessments even if they had discontinued the study drug.

Blood samples were collected from all randomized patients at the time of enrolment, at every scheduled clinical assessment and during relapses. To check individual compliance with intake, the fatty acids composition of patients' red blood cells' membranes was determined, by gas chromatography, according to a standard protocol. The fatty acid analyses were performed after study termination and thus did not influence the blinding.

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

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

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

Statistical analysis

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

Baseline characteristics were compared across all intervention groups by ANOVA or Kruskal-Wallis rank test for continuous variables and by mean Fisher's exact test for categorical variables, as appropriate.

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

For the secondary end-point outcome, the time to disability progression, Kaplan–Meier curves were constructed. Progression to disability and time thereof was compared in a pair-wise fashion for the active interventions versus placebo by the log-rank test in the main analysis and by Cox proportional-hazards models with adjustment for baseline EDSS score, age and DMT in the supportive analysis. Each test was performed with a significance level of

0.05. Multivariate models considered all variables with P <0.1 on univariate models. There was no overt violation of the proportionality assumption.

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

Role of the funding source

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

Results

Study population

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

Among the 80 patients, 20 patients were randomly assigned to each of the three groups to receive the interventions and 20 to receive placebo (Fig 1). Baseline characteristics of both the ITT and the per-protocol populations were similar across groups (Table 2A and 2B). All patients that drop-out completed follow-up until study completion and were included in the ITT analyses (Table 4). Five patients were lost to follow before their first scheduled visit and two other patients that dropped-out before their first scheduled visit progressed to secondary progressive MS. Fifteen patients dropped-out without successfully completing the "normalization" period including five pregnancies. Another 17 patients dropped-out early after entry baseline. Seven patients that dropped out were given monoclonal antibody treatment (natalizumab). Overall, a total of 41 (51%) patients completed the 42-month study (July 2007 through December 31st 2010, including the 12-month extended period) where one patient from group A and two from the placebo group transferred on natalizumab, and 39 (49%) patients either withdrew (drop-out) or lost to follow. Reasons for study-interventions discontinuation are listed in Figure 2.

Efficacy

Relapses

As a proof-of-concept trial we primarily needed to answer whether the interventions were effective for those MS patients who adhere to the assigned treatment, the per-protocol analysis.[45] For the sake of methodological comprehensiveness we also present the ITT analysis as a secondary analysis, to answer a different question, complementary to our core

hypothesis; like what happened to MS patients who were placed on the interventions (the effect of assignment).[45]

Regarding the per-protocol analysis, during the first year of treatment, the ARR was 0.80, 0.40, 0.78 and 0.83 for the four intervention groups respectively. During the second year, the ARR was 0.90, 0.40, 0.67 and 1.25 for the four intervention groups respectively. Overall, for the two-year primary end point, 8 relapses were recorded for the 10 patients in PLP10 group (0.40 ARR) versus 25 relapses for the 12 patients in placebo (1.04 ARR), a 64% adjusted relative rate reduction (RRR) for the PLP10 group (RRR 0.36, 95% confidence interval (CI) 0.15 to 0.87, p=0.024) (Tables 3A, 5 and Fig 3A and 3C). Excluding 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, Tables 3B and 5). Pair-wise comparisons for the other two groups against placebo did not yield statistically significant results (Tables 3A, 3B). The proportion of patients with ≤1 relapse for the two years on-study was higher in the PLP10 group than in the placebo group (90% versus 42%, p=0.030, Table 5). Seeking to investigate further the observed difference, we compared the relapse rate during the 24 months before entry to the study to the 24 months on-treatment for each intervention group. We observed a statistically significant relative reduction in the ARR (70%) only in the PLP10 group (RRR 0.30; 95% CI 0.14 to 0.65, p=0.003, Table 3A); within-group comparisons for the three other groups ARR reduction was not significant and remained not significant when natalizumab treated patients were further excluded from the analysis. The effect of PLP10 through time at different time-windows versus placebo for all-time on-study patients is shown in Figures 3A to 3D. The ARR analysis, within time-windows, was not an assigned endpoint, but it could help in the process of evaluating parallel information as the time needed for a specific treatment intervention activity to be evident, as

well as the efficacy profile through time. PLP10 reached maximum effect within a year on-treatment (counting from the entry baseline) and remained stable at an ARR of 0.4, displaying a steadily reduced ARR with long free-relapse time-windows. These group B characteristics are considered important parameters of a successful MS treatment where the rule than the exception is the heterogeneity among patients' disease evolution. Specifically, Figure 3D demonstrates the dispersion of relapses throughout the 2-year period of all-time on-study (excluding patients on natalizumab) of PLP10 (n=10) versus placebo (n=10). Placebo, in line with the existing knowledge of how relapse history works in relation to future relapses on MS patients (contagion phenomenon) indicates the expected linearly increased trend of the relapse incidence.[46] The same phenomenon was true for the groups A and C. Finally, during the 12 month post-study extended period (January 1st 2010 to December 31st 2010) all-time on-study patients that received PLP10, showed persistent benefit in the ARR compared to placebo (six relapses for the 10 subjects within PLP10, 0.6 ARR versus 19 for the 12 subjects within placebo group, 1.58 ARR) indicating a statistically significant 62% adjusted relative rate reduction in the ARR for the PLP10 group (RRR 0.38, 95% CI 0.12 to 0.99, p=0.046).

Regarding the ITT analysis, within PLP10 group, none of the nine drop-out patients changed to natalizumab and two out of nine discontinued because of pregnancy, whereas two out of seven drop-out patients from the placebo group changed to natalizumab (a total of four patients within the placebo arm population were on natalizumab, including the two patients that transferred while all-time on-study versus none within PLP10 group (Supplementary 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). 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 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 patients on natalizumab, there was an increased statistically significant difference between the PLP10 and the placebo group for the same analysis (p=0.006) (Fig 4A). At two years, the cumulative probability of progression was 10% in the PLP10 group and 70% in the placebo group, which represents a decrease of 60 percentage points or a relative 86% decrease in the risk of sustained progression of disability within 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 difference was observed for any comparison of the other two groups compared to placebo (Fig 4A and Supplementary Information Fig 2).

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

represents a decrease of 25 percentage points or a relative 71% decrease of the PLP10 for the risk of sustained progression of disability (adjusted hazard ratio 0.22, 95% CI 0.04 to 1.07, p=0.06) (Fig 4B). Two versus seven out of the total randomized patients progressed to confirmed disability in the PLP10 and the placebo groups respectively. No significant differences were observed for groups A or C against placebo (Fig 4B). The mean change in Expanded Disability Status Scale (EDSS) score as a function of visit number is shown in Figure 5.

MRI

Over two years, the MRI results support the overall conclusion from the study that PLP10 has a positive effect on disease activity since only 29% from the PLP10 group as opposed to 67% from the placebo group developed new or enlarging T2 lesions (57% relative risk reduction). Excluding patients on natalizumab there is an increased relative risk reduction (64%) between PLP10 as opposed to placebo with 29% of patients on PLP10 and 80% on placebo with development of new or enlarging T2 lesions (Table 5).

Safety

Over the course of the 30 month study no significant adverse events were reported from any group. According to a questioner procedure the only aetiology for drop-outs was the palatability and smell of the formula preparations. Nausea was reported by two patients. No abnormal values observed on any of the biochemical and haematological blood tests. No allergic reactions reported.

Discussion

In this proof-of-concept clinical trial assessing the safety and efficacy of three cocktail intervention formulas in RRMS, we observed a significant benefit for the novel PLP10 intervention compared to placebo for both the ARR and the progression to disability. Our results include analyses pertaining to a total of 42 months study collected data, including the 12-month, free of intervention treatment, extension period. We focused on the per-protocol data analysis since it is the appropriate method to best provide the answer to the proof-of-concept trial-addressed question. The high drop-out rate was mostly the result of formulas palatability, a common phenomenon in trials using oily interventions where a lot of patients tend to drop-out soon after first dosage. We thus present our main per-protocol analysis, as well as a subgroup analysis excluding patients on natalizumab. We have found a statistically significant reduction in the ARR and the disability progression comparing not only patients on PLP10 versus placebo but also comparing the ARR of the PLP10 patients in the 24-month period prior to the study to the ARR of the 24 months on-study; the observed differences became larger when patients that received natalizumab (the most potent disease modifier) were excluded. The ARR decreased within a year on PLP10 and significantly remained stable until study completion. Statistically significant difference of ARR between patients on PLP10 versus placebo continued for the additional 12 month extended period (persistent effect) without significant difference on DMT. These clinical findings are supported by the results regarding the MRI analysis where the proportion of patients free from new or enlarging brain T2 lesions was also higher in PLP10 group versus placebo. The persistent effect within the extended period it is considered of major importance and supportive of the results since it is in agreement with the very long washouts, reported necessary, for omega-3 fatty acids and especially DHA to return towards pretreatment values within the fatty acids of plasma, platelets, monocytes and red blood cells.[42] This study also provides important 30-month,

placebo-controlled information about the safety of PLP10, A and C interventions. No severe side effects have been reported.

As medications used to treat MS become increasingly highly specific and potent, attention to safety is paramount. Current available treatments are products of reductionism, partially effective, associated with severe side effects without (re)myelinating or neuroprotective abilities. Interferons and glatiramer acetate, the most widely used first-line MS drugs available today, are associated with the least severe side-effects among MS therapies but they are reported with only 29-33% ARR reduction and with no significant effects on the progression of disability. Natalizumab as previously discussed and Fingolimod with 54% ARR reduction (without significant benefit on the progression of disability) are second-line drugs associated with severe side-effects.[47, 48]

No existing MS treatment has ever been designed as a result of SM concept approach or with a potential to effectively stimulate intrinsic remyelinating and neuroprotecting mechanisms or exert such an action. Now we propose that a holistic SM model approach has to be applied by synchronized action on all involved perturbed mechanisms. PLP10 has innovative characteristics with a postulated efficacy attained through different mechanisms of action and probably by the synergistic effect of its constituent ingredients. PLP10 has all the characteristics of a medical food with the action to feed a normal metabolic process by supplying nutritional structural membrane precursors, building blocks, and vitamins from dietary sources that enhance remyelination and neuroprotection and simultaneously promote normalization of all cellular membranes lipid content. The intention is to normalize the specific nutritional requirements of the MS patients.

Different factors and molecular entities appear to be part of the possible aetiology for MS with specific PUFA and antioxidants found to be key substances related to all known pathogenic and recovery mechanisms. But, it is well established that MS patients are characterized with significant deficiencies in antioxidant efficiency as well as in PUFA levels in blood and cellular membranes.[11, 49-51]

According to one hypothesis, the change in the ratio of omega-3 to omega-6, due to increasing consumption of omega-6 PUFA, meaning high accumulation of AA, in the Western diet, may be one of the major factors responsible for the increasing incidence of inflammatory diseases relative to populations. In the Western diet, the ratio of omega-3 to omega-6 is about 1:20–30; in populations that consume fish-based diets, the ratio is about 1:1–2.[52, 53] The intervention daily dose was aiming and believed to be high enough to restore/amplify body efficient antioxidant activity and ensure cellular membranes lipid profile normalization (PUFA content) and simultaneously potentiate involvement of the ingredients in the anti-inflammatory and recovery mechanisms. Diet fatty acid molecules need about six months period to exert their beneficial effect and this essential parameter was for the first time under consideration in our study design (normalization period).[42] This chronotherapy parameter it is of major importance in line with the SM treatment philosophy and if it is not included in the trial design the possibility of misleading result evaluation greatly increases. In fact, considering that omega-3 supplementation can release and replace excess AA within the cellular membranes, we can speculate that an increased inflammatory activity can possibly result during the first six months of supplementation (during normalization period).

The maintenance of myelin requires continued turnover of its components throughout life.[54,55] In addition to the EPA, DHA, LA, and GLA, PLP10 was containing limited quantities of

other structural PUFA, specific MUFA (mostly oleic acid) and SFA (palmitic and stearic acid), specifically to provide a direct source for neuronal cell membranes rehabilitation and for (re)myelination and neuroprotection since they are all major components, precursors and building blocks of any new physiological myelin and cellular membranes in general. Assembly of the correct molecules into myelin membrane may be especially critical during active synthesis. Possibly, if critical constituents aren't available or are metabolically blocked, amyelination, dysmyelination or demyelination may ensue.[56]

The well known and established safety of the ingredients used and the protocol guidelines were supportive reasons for us to proceed with the clinical study even though with limitation on the pre-estimation of required trial sample size as it was discussed in method section. The adherence of the subjects is another issue but the duration of the study (42 months) is adding power to the results;[44] having the research questions been consciously and carefully approached and answered. Furthermore, the statistical methodologies used along with the appropriate adjustments, broadly accepted for MS clinical trials, power the findings, results, and significance. The baseline characteristics of the treatment arms could possibly be considered indicative of four very active groups of patients but that was the result of the limited number of RRMS population eligible for the study within Cyprus. On the other hand the balanced baseline characteristics without statistical differences, the statistical adjustments (for all important baseline parameters i.e. relapses, EDSS, age and DMT) and the randomization within four different groups are the safety valves against data misinterpretation. Yet, in small randomized control trials with a high drop-out rate, the per-protocol analysis could be affected by the characteristics of the patients dropping out. In order to safeguard our findings in the best possible way under the circumstances, we proceeded to adjusting for confounders. Moreover, we cannot discard our finding as a false

positive, given that this is a randomized, double-blind, placebo-controlled clinical trial and, despite its small sample size, represents a piece of evidence that only a larger randomized controlled trial can replicate or refute. It is possible to question why DMTs efficacy cannot be emerged out of the data analysis, of the four treatment arms, and in accordance to their published values. We believe that the limited efficacy of the DMTs, the sample size and the statistical adjustments were strong limiting determining factors for such an indication to be countable. An additional argument is that the efficacy reported for the analysis of pre-treatment (24 months before entry baseline) versus on-trial ARR could be considered as potentially biased due to differences of how relapses were defined during the course of a study compared to pre-treatment period; or due to regression to the mean or placebo effect. This analysis was performed as an additional exploratory analysis that we were able to do due to the availability of data. The relapses of the two pre-treatment years were drawn out of the patients' archival records by the same treating neurologist involved in the study (MP), and according to the patients' hospitalization date for receiving intravenous methyl-prednisolone. This analysis was not used as a primary or a secondary end-point under investigation although it is usually reported by many clinical studies. As a matter of fact many early phase trials are based only on such an analysis (before versus after treatment results). In almost all MS trials the number of relapses within the two years before baseline is a factor under adjustment for the statistical analyses.[48] The inclusion of the post-hoc MRI analysis is another limiting factor that needs attention since it was used as an additional aside exploratory approach (due to study budget limitations it was not possible to be used as a formal endpoint); but the MRI evaluation was blinded and can be considered as representative of the randomized subjects within the treatment arms. As far as the regression to the mean and the placebo effect concerns we believe that the 6-month normalization period is an accountable and valuable eliminating factor of the possible effect; as well as the presence of four groups,

where only the PLP10 treatment arm is associated with statistically significant efficacy versus placebo.

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

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

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

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.

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.

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

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.

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.

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 1year**	**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 by1 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 by1 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
† 1out 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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Ethical approval: Cyprus National bioethics committee (reference ΕΕΒΚ/ΕΠ/2005/10).

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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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Figure legends

Figure 1. Study Flowchart

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

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

Abbreviations: PL, phospholipid; IFN-γ, interferon γ; IL-2, interleukin 2; NFκB, nuclear factor kappa B; PGE2, prostaglandin E2; PPARγ, peroxisome proliferator-activated receptor γ; PUFAs, polyunsaturated fatty acids; TGFβ, 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.

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. **

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

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

Panel D demonstrates the dispersion of relapses throughout the 2-year period of all-time on-study (excluding patients on natalizumab) of PLP10 (n=10) *vs.* placebo (n=10). Placebo

shows an irregular dispersion of relapses in relation to PLP10 group with linear increasing trend wile PLP10 shows a stabilized linear trend. By using the per-protocol model where patients on natalizumab were excluded, we could compare the number of relapses on a same number of patients.

** Including the patients on natalizumab.

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

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

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

¶ Including patients on natalizumab

¶¶ Excluding patients on natalizumab

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

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Abstract

Objective To assess whether our three novel interventions, formulated based on systems 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.

Results A total of 41 (51%) patients completed the 42-month trial. Overall, 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). No adverse events were reported. Interventions A (10 patients) and C (9 patients) showed no significant efficacy.

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

Trial registration International Standard Randomized Controlled Trial, number ISRCTN87818535.

Introduction

Multiple sclerosis (MS) is a complex multifactorial disease that results from interplay between as yet unidentified environmental factors and susceptibility genes.[1-3] Together, these factors trigger a cascade of events, involving engagement of the immune system, inflammatory injury of myelin, axons and glia, functional recovery and structural repair, gliosis, and neurodegeneration.[4] The bio-mechanisms involved are: immune-mediated inflammation, oxidative stress and excitotoxicity.[5-9] These mechanisms may all contribute to oligodendrocyte and neuronal damage and even cell death, hence promoting disease progression. The increasing prevalence of 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.

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

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

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

In vitro and in vivo studies have demonstrated that dietary EPA, DHA, LA, and GLA can be implicated and modulate almost all known complex network of events and pathways repertoire in MS pathophysiology. Brain membrane fatty acid composition can be modified with dietary supplementation, but the process has been showed to be age dependent (it takes much longer in adults versus developing brains) as well as possibly dependent on the quantities of the dietary/supplemented PUFAs.[14] Both human and animal studies proved that diets high in DHA and EPA increase the proportion of these PUFA in the membranes of inflammatory cells and reduce the levels of AA.[12, 15] The anti-inflammatory properties of

omega-3 include production of PGs and TXs of the 3-series and LTs of the 5-series (Fig 2).[14, 16] Resolvins and protectins are biosynthesized from omega-3 fatty acids via cyclooxygenase-2/lipoxygenase (COX-2/LOX) pathways and they promote control of inflammation in neural tissues (Fig 2).[17-21] T-cell proliferation in acute and chronic inflammation can be reduced by supplementation with either omega-6 or omega-3 PUFA.[22] Furthermore, vitamin E is an important antioxidant that can interrupt the propagation of free radical chain reactions.[23] Vitamin E (alpha-tocopherol, an isoform of vitamin E) efficiently detoxifies hydroxyl, perhydroxyl and superoxide free radicals.[24] However γ-tocopherol (another isoform of vitamin E) seems to be more efficiently implicated in trapping NO radicals.[25] In addition alpha–tocopherol exerts non-antioxidant properties, including modulation of cell signaling and immune function, regulation of transcription, and induction of apoptosis.[26]

Moreover, omega-3 fatty acids electrophilic derivatives formed by COX-2, in activated macrophages can stimulate the nuclear respiratory factor (Nrf2) inducing the transcription of neuroprotective and antioxidant related genes, and can activate the peroxisome proliferator-activated receptor (PPAR)γ for anti-inflammatory response.[27-29] In animal studies, EPA and DHA proved to be endogenous ligands of RXRs, with positive effect on neurogenesis.[30] Additionaly, in 2008 Salvati and coworkers reported evidense of accelerated myelination in DHA- and EPA-treated animals.[32] Moreover, DHA and EPA are reported to significantly decrease the levels of metalloproteinases (MMP) -2, -3, -9, and -13 with a significant role in the migration of lymphocytes into the CNS by inducing disruption of the blood brain barrier (BBB), an important step in the formation of MS lesions.[33-39]

Based on the above, specific PUFA and antioxidant vitamins fulfill the criterion of biologic plausibility and have the potential to diminish MS symptoms severity and activity, even

promoting recovery (remyelination).[11] Overall, PLP10 contains multiple ingredients (omega-3, omega-6 and other fatty acids and vitamins) potentially able to modulate key interconnected components (i.e. genes, proteins) and structural molecules (i.e. cellular membrane lipids, receptors) within the functional network of events of MS pathogenesis.[40]

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

Methods

Patients

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

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

The study was conducted in accordance with the standards of the International Conference of Harmonization Guidelines for Good Clinical Practice. The protocol was developed by the investigators and it was approved by the Cyprus National Bioethics Committee and was overseen by an independent safety-monitoring committee evaluating the safety and over-all benefit-risk profiles. The adherence of care providers with the protocol was assessed by an external committee assigned by the funder of the project through reviews of case report forms. All patients gave written informed consent at the time of enrolment.

Randomization and masking

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

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

the conduct of the study were blinded throughout the study. Treating/examining physician, other investigators, pharmacist, neuroradiologist and patients were masked to treatment allocation.

Procedures and end points

The specific omega-3 (re-esterified glycerides) and omega-6 (glycerides) raw materials were purchased according to the required interventions' PUFA-fraction specification (molecular structure, quantity/ratio and quality) with vitamin E (alpha-tocopherol) used as antioxidant stabilizer by the supplier. The vitamins and masking aroma were purchased separately. The mixing of fractions to the final required intervention-composition specification was always performed by the same team of scientists under the supervision of the involved medical biochemist and lipidology specialist, under appropriate conditions every six months. Interventions were stored refrigerated in dark until use. See Table 1 and Supplementary Information Methods 1 and 2 for intervention specification detailed description and study/intervention rational.

Participants were randomly assigned to receive: in group A, a daily dose of a 19.5ml mixture of 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 A); in group B PLP10, a daily dose of a 19.5ml mixture of 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 pure γ-tocopherol (760mg) plus citrus-aroma (intervention B); in group C, a daily dose of a 19.5ml mixture of pure γ-tocopherol (760mg) dispersed in pure virgin olive oil (16,137 mg) plus citrus-aroma

(intervention C) and in group D placebo, a daily dose of a 19.5ml mixture of pure virgin olive oil (16,930mg) plus citrus-aroma (intervention D) (Table 1). The institution's pharmacist was responsible for the appropriate storage and handling of the interventions to the individual participants. The interventions were taken orally once daily 30 minutes before dinner by a dosage calibrated cup for 30 months. The ingredients, ratio and dose have been selected based on their biophysical interrelation to the total known multiple MS causing factors, their biochemical importance and the role expected to play in the normalisation and treatment of the involved complex network of events in the disease pathophysiology. Moreover, the high intake dosage was used to overcome any abnormal dietary accumulation of related agents as a result of patients' food intake habits, irrespective of geographical origin, in relation to the daily consumption ratio of the total fatty acid intake; in order to end-up with omega-3 to omega-6 PUFA indicated physiological body ratio composition of 1:1 wt/wt.

The period beginning from July 1st 2007 (enrolment) until December 31st 2007 (entry baseline) was used for normalization period. This six-month normalization period would allow the interventions' agents to exert their beneficial effect (for the incorporation/normalization of cell membranes by oral PUFA, since they need four to six months to exert pivotal action on immune and neural cells, correction of antioxidant deficiency and body PUFA, and optimal normalization of EPA and DHA levels/ratio).[41-43] The study was completed on December 31st 2009 and the recording of relapses continued until December 31st 2010. More clearly the study included the "normalization period" (July 1st 2007 to Dec 31st 2007), the "on treatment" period (Jan 1st 2008 to Dec 31st 2009) and the 12-month "extended period" (Jan 1st 2010– Dec 31st 2010).

Depending on their clinical status and in accordance with the ethical issues governing clinical trials participants continued receiving the indicative regular available treatments, according to international guidelines with persistent evaluation of any side-effects and adverse events. The study was designed to end 30 months after enrolment and clinical assessments were scheduled at entry baseline, 3, 9, 15, 21 and 24 months on-treatment. Patients were also clinically examined by the treating neurologist within 48 hours after the onset of new or recurrent neurologic symptoms.

The primary end point was the ARR at two years. A relapse was defined as new or recurrent neurologic symptoms not associated with fever or infection that lasted for at least 24 hours and accompanied by new neurologic signs. Relapses were treated with methyl-prednisolone at a dose of 1g intravenous per day, for three days followed by prednisone orally at a dose of 1mg/kg of weight per day on a tapering scheme for three weeks. The secondary end point at two years was the time to confirmed disability progression, defined as an increase of 1.0 or more on EDSS, confirmed after six months (progression could not be confirmed during a relapse). The final EDSS score was confirmed six months after the end of the study. A post-hoc analysis was performed assessing the proportion of patients free from new or enlarging T2 lesions on brain MRI scans at the end of the study for the per-protocol participants of the group receiving the highest effective intervention versus placebo. Comparison was made only versus the available archival MRI scans up to three months before the enrolment date. MRI scans were performed and blindly analyzed at an MRI evaluation centre. The patients continued to be followed for additional 12 months after completion of the trial and relapses were recorded. Finally, patients were strongly encouraged to remain in the study for follow-up assessments even if they had discontinued the study drug.

Blood samples were collected from all randomized patients at the time of enrolment, at every scheduled clinical assessment and during relapses. To check individual compliance with intake, the fatty acids composition of patients' red blood cells' membranes was determined, by gas chromatography, according to a standard protocol. The fatty acid analyses were performed after study termination and thus did not influence the blinding.

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

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

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

Statistical analysis

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

Baseline characteristics were compared across all intervention groups by ANOVA or Kruskal-Wallis rank test for continuous variables and by mean Fisher's exact test for categorical variables, as appropriate.

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

For the secondary end-point outcome, the time to disability progression, Kaplan–Meier curves were constructed. Progression to disability and time thereof was compared in a pair-wise fashion for the active interventions versus placebo by the log-rank test in the main analysis and by Cox proportional-hazards models with adjustment for baseline EDSS score, age and DMT in the supportive analysis. Each test was performed with a significance level of

0.05. Multivariate models considered all variables with P <0.1 on univariate models. There was no overt violation of the proportionality assumption.

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

Role of the funding source

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

Results

Study population

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

Among the 80 patients, 20 patients were randomly assigned to each of the three groups to receive the interventions and 20 to receive placebo (Fig 1). Baseline characteristics of both the ITT and the per-protocol populations were similar across groups (Table 2A and 2B). All patients that drop-out completed follow-up until study completion and were included in the ITT analyses (Table 4). Five patients were lost to follow before their first scheduled visit and two other patients that dropped-out before their first scheduled visit progressed to secondary progressive MS. Fifteen patients dropped-out without successfully completing the "normalization" period including five pregnancies. Another 17 patients dropped-out early after entry baseline. Seven patients that dropped out were given monoclonal antibody treatment (natalizumab). Overall, a total of 41 (51%) patients completed the 42-month study (July 2007 through December 31st 2010, including the 12-month extended period) where one patient from group A and two from the placebo group transferred on natalizumab, and 39 (49%) patients either withdrew (drop-out) or lost to follow. Reasons for study-interventions discontinuation are listed in Figure 2.

Efficacy

Relapses

As a proof-of-concept trial we primarily needed to answer whether the interventions were effective for those MS patients who adhere to the assigned treatment, the per-protocol analysis.[45] For the sake of methodological comprehensiveness we also present the ITT analysis as a secondary analysis, to answer a different question, complementary to our core

hypothesis; like what happened to MS patients who were placed on the interventions (the effect of assignment).[45]

Regarding the per-protocol analysis, during the first year of treatment, the ARR was 0.80, 0.40, 0.78 and 0.83 for the four intervention groups respectively. During the second year, the ARR was 0.90, 0.40, 0.67 and 1.25 for the four intervention groups respectively. Overall, for the two-year primary end point, 8 relapses were recorded for the 10 patients in PLP10 group (0.40 ARR) versus 25 relapses for the 12 patients in placebo (1.04 ARR), a 64% adjusted relative rate reduction (RRR) for the PLP10 group (RRR 0.36, 95% confidence interval (CI) 0.15 to 0.87, p=0.024) (Tables 3A, 5 and Fig 3A and 3C). Excluding 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, Tables 3B and 5). Pair-wise comparisons for the other two groups against placebo did not yield statistically significant results (Tables 3A, 3B). The proportion of patients with ≤1 relapse for the two years on-study was higher in the PLP10 group than in the placebo group (90% versus 42%, p=0.030, Table 5). Seeking to investigate further the observed difference, we compared the relapse rate during the 24 months before entry to the study to the 24 months on-treatment for each intervention group. We observed a statistically significant relative reduction in the ARR (70%) only in the PLP10 group (RRR 0.30; 95% CI 0.14 to 0.65, p=0.003, Table 3A); within-group comparisons for the three other groups ARR reduction was not significant and remained not significant when natalizumab treated patients were further excluded from the analysis. The effect of PLP10 through time at different time-windows versus placebo for all-time on-study patients is shown in Figures 3A to 3D. The ARR analysis, within time-windows, was not an assigned endpoint, but it could help in the process of evaluating parallel information as the time needed for a specific treatment intervention activity to be evident, as

well as the efficacy profile through time. PLP10 reached maximum effect within a year on-treatment (counting from the entry baseline) and remained stable at an ARR of 0.4, displaying a steadily reduced ARR with long free-relapse time-windows. These group B characteristics are considered important parameters of a successful MS treatment where the rule than the exception is the heterogeneity among patients' disease evolution. Specifically, Figure 3D demonstrates the dispersion of relapses throughout the 2-year period of all-time on-study (excluding patients on natalizumab) of PLP10 (n=10) versus placebo (n=10). Placebo, in line with the existing knowledge of how relapse history works in relation to future relapses on MS patients (contagion phenomenon) indicates the expected linearly increased trend of the relapse incidence.[46] The same phenomenon was true for the groups A and C. Finally, during the 12 month post-study extended period (January 1st 2010 to December 31st 2010) all-time on-study patients that received PLP10, showed persistent benefit in the ARR compared to placebo (six relapses for the 10 subjects within PLP10, 0.6 ARR versus 19 for the 12 subjects within placebo group, 1.58 ARR) indicating a statistically significant 62% adjusted relative rate reduction in the ARR for the PLP10 group (RRR 0.38, 95% CI 0.12 to 0.99, p=0.046).

Regarding the ITT analysis, within PLP10 group, none of the nine drop-out patients changed to natalizumab and two out of nine discontinued because of pregnancy, whereas two out of seven drop-out patients from the placebo group changed to natalizumab (a total of four patients within the placebo arm population were on natalizumab, including the two patients that transferred while all-time on-study versus none within PLP10 group (Supplementary 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). 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 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 patients on natalizumab, there was an increased statistically significant difference between the PLP10 and the placebo group for the same analysis (p=0.006) (Fig 4A). At two years, the cumulative probability of progression was 10% in the PLP10 group and 70% in the placebo group, which represents a decrease of 60 percentage points or a relative 86% decrease in the risk of sustained progression of disability within 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 difference was observed for any comparison of the other two groups compared to placebo (Fig 4A and Supplementary Information Fig 2).

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

represents a decrease of 25 percentage points or a relative 71% decrease of the PLP10 for the risk of sustained progression of disability (adjusted hazard ratio 0.22, 95% CI 0.04 to 1.07, p=0.06) (Fig 4B). Two versus seven out of the total randomized patients progressed to confirmed disability in the PLP10 and the placebo groups respectively. No significant differences were observed for groups A or C against placebo (Fig 4B). The mean change in Expanded Disability Status Scale (EDSS) score as a function of visit number is shown in Figure 5.

MRI

Over two years, the MRI results support the overall conclusion from the study that PLP10 has a positive effect on disease activity since only 29% from the PLP10 group as opposed to 67% from the placebo group developed new or enlarging T2 lesions (57% relative risk reduction). Excluding patients on natalizumab there is an increased relative risk reduction (64%) between PLP10 as opposed to placebo with 29% of patients on PLP10 and 80% on placebo with development of new or enlarging T2 lesions (Table 5).

Safety

Over the course of the 30 month study no significant adverse events were reported from any group. According to a questioner procedure the only aetiology for drop-outs was the palatability and smell of the formula preparations. Nausea was reported by two patients. No abnormal values observed on any of the biochemical and haematological blood tests. No allergic reactions reported.

Discussion

In this proof-of-concept clinical trial assessing the safety and efficacy of three cocktail intervention formulas in RRMS, we observed a significant benefit for the novel PLP10 intervention compared to placebo for both the ARR and the progression to disability. Our results include analyses pertaining to a total of 42 months study collected data, including the 12-month, free of intervention treatment, extension period. We focused on the per-protocol data analysis since it is the appropriate method to best provide the answer to the proof-of-concept trial-addressed question. The high drop-out rate was mostly the result of formulas palatability, a common phenomenon in trials using oily interventions where a lot of patients tend to drop-out soon after first dosage. We thus present our main per-protocol analysis, as well as a subgroup analysis excluding patients on natalizumab. We have found a statistically significant reduction in the ARR and the disability progression comparing not only patients on PLP10 versus placebo but also comparing the ARR of the PLP10 patients in the 24-month period prior to the study to the ARR of the 24 months on-study; the observed differences became larger when patients that received natalizumab (the most potent disease modifier) were excluded. The ARR decreased within a year on PLP10 and significantly remained stable until study completion. Statistically significant difference of ARR between patients on PLP10 versus placebo continued for the additional 12 month extended period (persistent effect) without significant difference on DMT. These clinical findings are supported by the results regarding the MRI analysis where the proportion of patients free from new or enlarging brain T2 lesions was also higher in PLP10 group versus placebo. The persistent effect within the extended period it is considered of major importance and supportive of the results since it is in agreement with the very long washouts, reported necessary, for omega-3 fatty acids and especially DHA to return towards pretreatment values within the fatty acids of plasma, platelets, monocytes and red blood cells.[42] This study also provides important 30-month,

placebo-controlled information about the safety of PLP10, A and C interventions. No severe side effects have been reported.

As medications used to treat MS become increasingly highly specific and potent, attention to safety is paramount. Current available treatments are products of reductionism, partially effective, associated with severe side effects without (re)myelinating or neuroprotective abilities. Interferons and glatiramer acetate, the most widely used first-line MS drugs available today, are associated with the least severe side-effects among MS therapies but they are reported with only 29-33% ARR reduction and with no significant effects on the progression of disability. Natalizumab as previously discussed and Fingolimod with 54% ARR reduction (without significant benefit on the progression of disability) are second-line drugs associated with severe side-effects.[47, 48]

No existing MS treatment has ever been designed as a result of SM concept approach or with a potential to effectively stimulate intrinsic remyelinating and neuroprotecting mechanisms or exert such an action. Now we propose that a holistic SM model approach has to be applied by synchronized action on all involved perturbed mechanisms. PLP10 has innovative characteristics with a postulated efficacy attained through different mechanisms of action and probably by the synergistic effect of its constituent ingredients. PLP10 has all the characteristics of a medical food with the action to feed a normal metabolic process by supplying nutritional structural membrane precursors, building blocks, and vitamins from dietary sources that enhance remyelination and neuroprotection and simultaneously promote normalization of all cellular membranes lipid content. The intention is to normalize the specific nutritional requirements of the MS patients.

Different factors and molecular entities appear to be part of the possible aetiology for MS with specific PUFA and antioxidants found to be key substances related to all known pathogenic and recovery mechanisms. But, it is well established that MS patients are characterized with significant deficiencies in antioxidant efficiency as well as in PUFA levels in blood and cellular membranes.[11, 49-51]

According to one hypothesis, the change in the ratio of omega-3 to omega-6, due to increasing consumption of omega-6 PUFA, meaning high accumulation of AA, in the Western diet, may be one of the major factors responsible for the increasing incidence of inflammatory diseases relative to populations. In the Western diet, the ratio of omega-3 to omega-6 is about 1:20–30; in populations that consume fish-based diets, the ratio is about 1:1–2.[52, 53] The intervention daily dose was aiming and believed to be high enough to restore/amplify body efficient antioxidant activity and ensure cellular membranes lipid profile normalization (PUFA content) and simultaneously potentiate involvement of the ingredients in the anti-inflammatory and recovery mechanisms. Diet fatty acid molecules need about six months period to exert their beneficial effect and this essential parameter was for the first time under consideration in our study design (normalization period).[42] This chronotherapy parameter it is of major importance in line with the SM treatment philosophy and if it is not included in the trial design the possibility of misleading result evaluation greatly increases. In fact, considering that omega-3 supplementation can release and replace excess AA within the cellular membranes, we can speculate that an increased inflammatory activity can possibly result during the first six months of supplementation (during normalization period).

The maintenance of myelin requires continued turnover of its components throughout life.[54,55] In addition to the EPA, DHA, LA, and GLA, PLP10 was containing limited quantities of

other structural PUFA, specific MUFA (mostly oleic acid) and SFA (palmitic and stearic acid), specifically to provide a direct source for neuronal cell membranes rehabilitation and for (re)myelination and neuroprotection since they are all major components, precursors and building blocks of any new physiological myelin and cellular membranes in general. Assembly of the correct molecules into myelin membrane may be especially critical during active synthesis. Possibly, if critical constituents aren't available or are metabolically blocked, amyelination, dysmyelination or demyelination may ensue.[56]

The well known and established safety of the ingredients used and the protocol guidelines were supportive reasons for us to proceed with the clinical study even though with limitation on the pre-estimation of required trial sample size as it was discussed in method section. The adherence of the subjects is another issue but the duration of the study (42 months) is adding power to the results;[44] having the research questions been consciously and carefully approached and answered. Furthermore, the statistical methodologies used along with the appropriate adjustments, broadly accepted for MS clinical trials, power the findings, results, and significance. The baseline characteristics of the treatment arms could possibly be considered indicative of four very active groups of patients but that was the result of the limited number of RRMS population eligible for the study within Cyprus. On the other hand the balanced baseline characteristics without statistical differences, the statistical adjustments (for all important baseline parameters i.e. relapses, EDSS, age and DMT) and the randomization within four different groups are the safety valves against data misinterpretation. Yet, in small randomized control trials with a high drop-out rate, the per-protocol analysis could be affected by the characteristics of the patients dropping out. In order to safeguard our findings in the best possible way under the circumstances, we proceeded to adjusting for confounders. Moreover, we cannot discard our finding as a false

positive, given that this is a randomized, double-blind, placebo-controlled clinical trial and, despite its small sample size, represents a piece of evidence that only a larger randomized controlled trial can replicate or refute. It is possible to question why DMTs efficacy cannot be emerged out of the data analysis, of the four treatment arms, and in accordance to their published values. We believe that the limited efficacy of the DMTs, the sample size and the statistical adjustments were strong limiting determining factors for such an indication to be countable. An additional argument is that the efficacy reported for the analysis of pre-treatment (24 months before entry baseline) versus on-trial ARR could be considered as potentially biased due to differences of how relapses were defined during the course of a study compared to pre-treatment period; or due to regression to the mean or placebo effect. This analysis was performed as an additional exploratory analysis that we were able to do due to the availability of data. The relapses of the two pre-treatment years were drawn out of the patients' archival records by the same treating neurologist involved in the study (MP), and according to the patients' hospitalization date for receiving intravenous methyl-prednisolone. This analysis was not used as a primary or a secondary end-point under investigation although it is usually reported by many clinical studies. As a matter of fact many early phase trials are based only on such an analysis (before versus after treatment results). In almost all MS trials the number of relapses within the two years before baseline is a factor under adjustment for the statistical analyses.[48] The inclusion of the post-hoc MRI analysis is another limiting factor that needs attention since it was used as an additional aside exploratory approach (due to study budget limitations it was not possible to be used as a formal endpoint); but the MRI evaluation was blinded and can be considered as representative of the randomized subjects within the treatment arms. As far as the regression to the mean and the placebo effect concerns we believe that the 6-month normalization period is an accountable and valuable eliminating factor of the possible effect; as well as the presence of four groups,

where only the PLP10 treatment arm is associated with statistically significant efficacy versus placebo.

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

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

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

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.

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.

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

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.

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.

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 1year**	**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 by1 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 by1 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
† 1out 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 for the protocol and study design. M.C.P was the evaluator of patients' clinical progress signs and the treatment physician. I.S.P and G.N.L were involved in the non-pharmacologic treatment-related care. I.S.P performed the literature search and both I.S.P and G.N.L contributed on the intervention formulation and composition rational. I.S.P supervised the composition procedure of the interventions and the fatty acid profile analysis of the red blood cell membranes. E.E.N and I.S.P performed the statistical analyses. E.E.N was an independent scientist. All authors vouch for the accuracy and completeness of the data and the statistical analyses.

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Ethical approval: Cyprus National bioethics committee (reference ΕΕΒΚ/ΕΠ/2005/10).

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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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Figure legends

Figure 1. Study Flowchart

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

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

Abbreviations: PL, phospholipid; IFN-γ, interferon γ; IL-2, interleukin 2; NFκB, nuclear factor kappa B; PGE2, prostaglandin E2; PPARγ, peroxisome proliferator-activated receptor γ; PUFAs, polyunsaturated fatty acids; TGFβ, 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.

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. **

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

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

Panel D demonstrates the dispersion of relapses throughout the 2-year period of all-time on-study (excluding patients on natalizumab) of PLP10 (n=10) *vs.* placebo (n=10). Placebo

shows an irregular dispersion of relapses in relation to PLP10 group with linear increasing trend wile PLP10 shows a stabilized linear trend. By using the per-protocol model where patients on natalizumab were excluded, we could compare the number of relapses on a same number of patients.

** Including the patients on natalizumab.

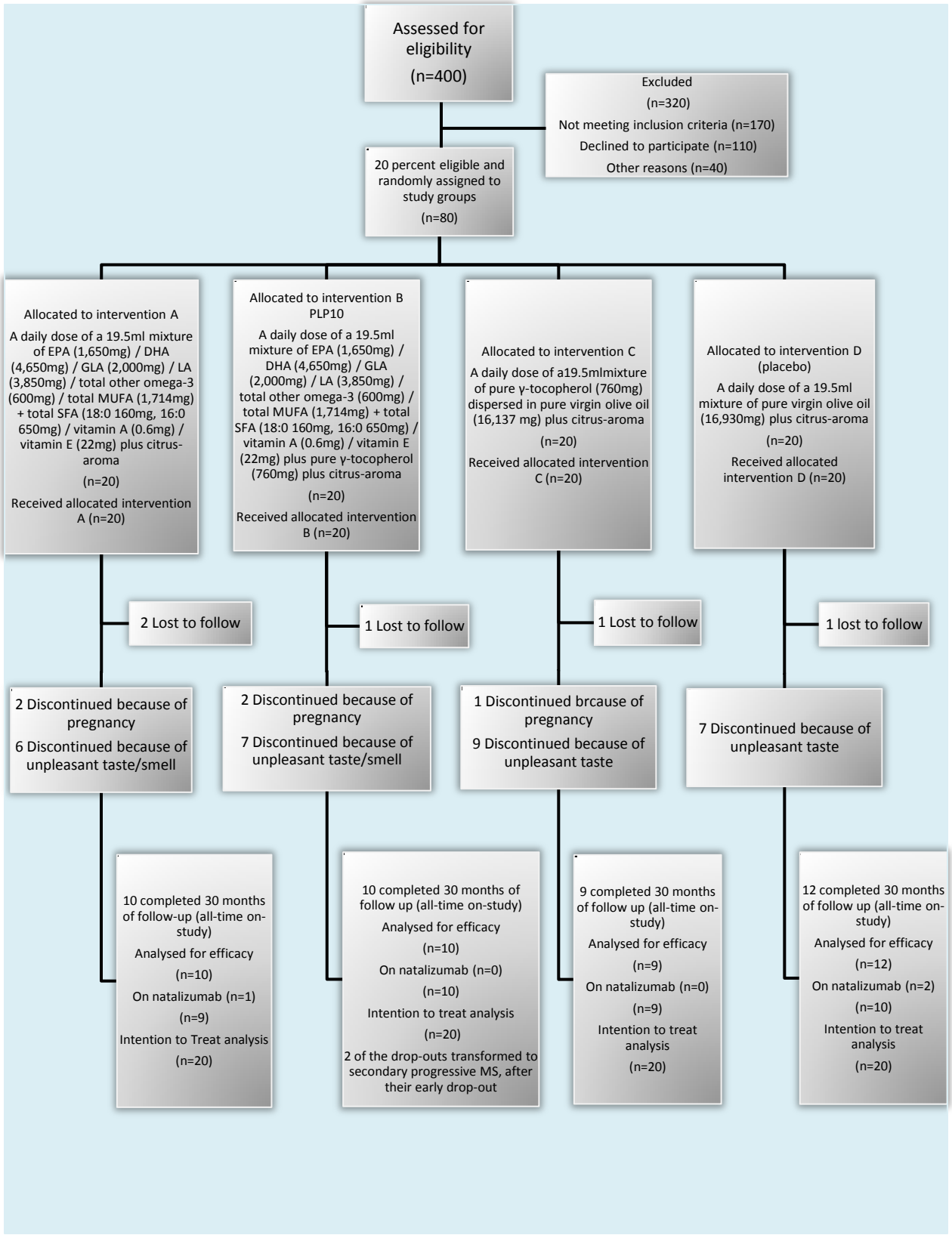
Figure 4. Panel 4A demonstrates the Kaplan–Meier plot of the time to sustained progression of disability among all-time on-study patients, excluding patients on natalizumab, receiving intervention A, PLP10 and C as compared with placebo. PLP10 reduced the risk of sustained progression of disability by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

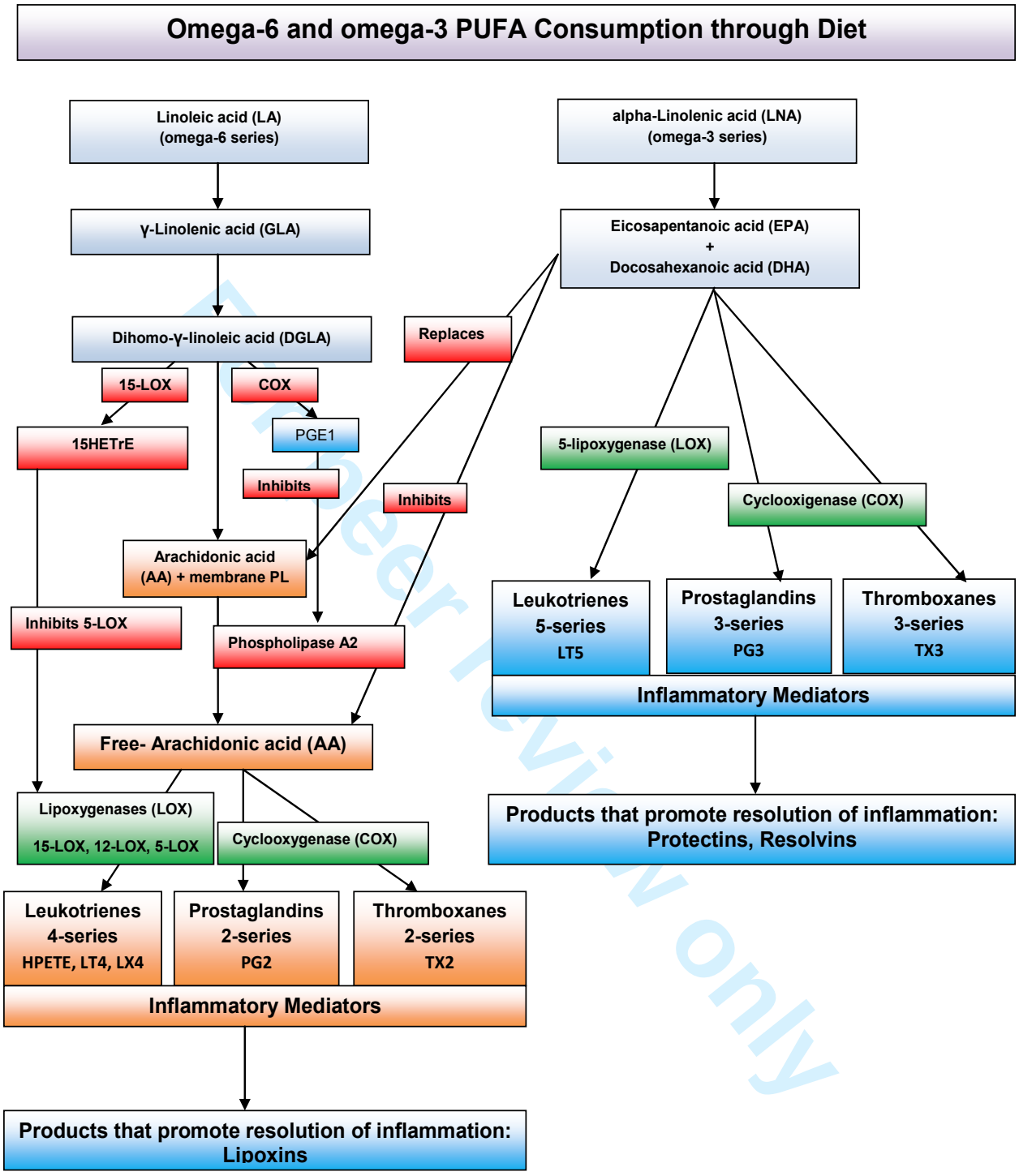
Panel 4B demonstrates the Kaplan–Meier plot of the time to sustained progression of disability among ITT population receiving intervention A, PLP10 and C as compared with placebo. PLP10 reduced the risk of sustained progression of disability by 71% over two years (p=0.052, trend). Intervention formula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

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

¶ Including patients on natalizumab

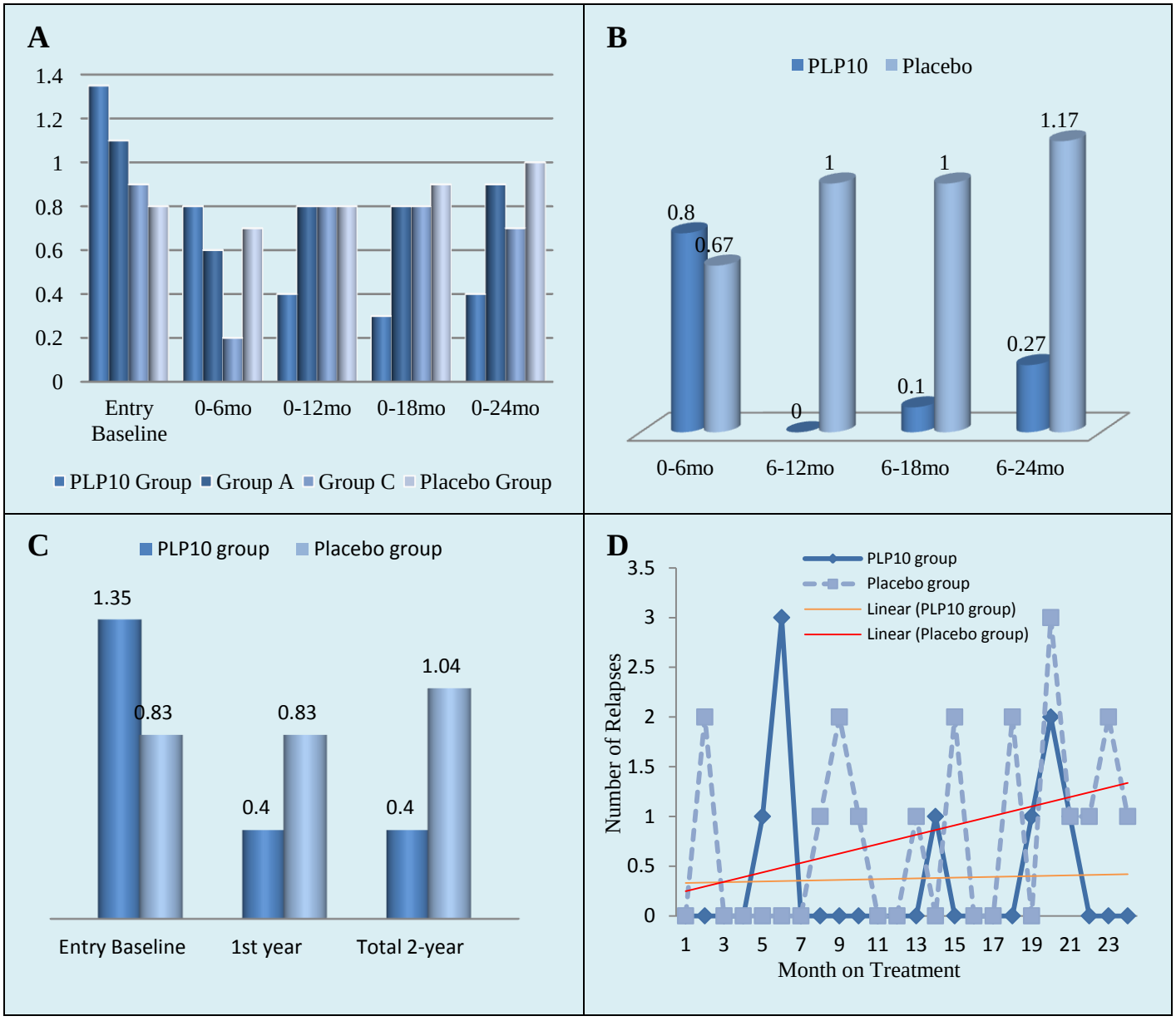
¶¶ Excluding patients on natalizumab

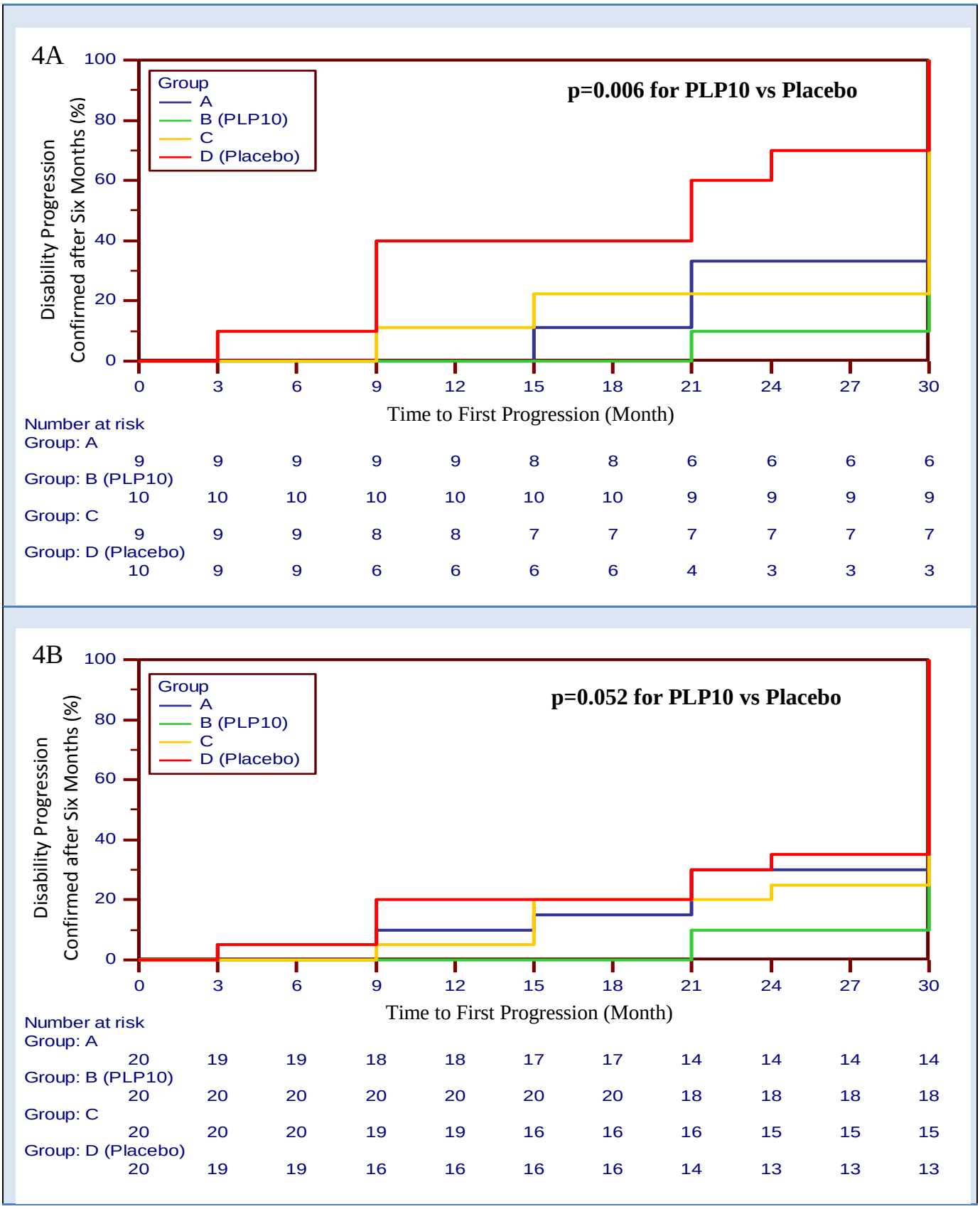

Assessed for eligibility
(n=400)
Excluded
(n=320)
Not meeting inclusion criteria (n=170)
Declined to participate (n=110)
Other reasons (n=40)
20 percent eligible and randomly assigned to study groups
(n=80)
Allocated to intervention A
A daily dose of a 19.5ml mixture of EPA (1,650mg) / DHA (4,650mg) / 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
(n=20)
Received allocated intervention A (n=20)
Allocated to intervention B PLP10
A daily dose of a 19.5ml mixture of 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 pure γ-tocopherol (760mg) plus citrus-aroma
(n=20)
Received allocated intervention B (n=20)
Allocated to intervention C
A daily dose of a19.5mlmixture of pure γ-tocopherol (760mg) dispersed in pure virgin olive oil (16,137 mg) plus citrus-aroma
(n=20)
Received allocated intervention C (n=20)
Allocated to intervention D (placebo)
A daily dose of a 19.5ml mixture of pure virgin olive oil (16,930mg) plus citrus-aroma
(n=20)
Received allocated intervention D (n=20)
2 Lost to follow
1 Lost to follow
1 Lost to follow
1 lost to follow
2 Discontinued because of pregnancy
6 Discontinued because of unpleasant taste/smell
2 Discontinued because of pregnancy
7 Discontinued because of unpleasant taste/smell
1 Discontinued brcause of pregnancy
9 Discontinued because of unpleasant taste
7 Discontinued because of unpleasant taste
10 completed 30 months of follow-up (all-time on-study)
Analysed for efficacy
(n=10)
On natalizumab (n=1)
(n=9)
Intention to Treat analysis
(n=20)
10 completed 30 months of follow up (all-time on-study)
Analysed for efficacy
(n=10)
On natalizumab (n=0)
(n=10)
Intention to treat analysis
(n=20)
2 of the drop-outs transformed to secondary progressive MS, after their early drop-out
9 completed 30 months of follow up (all-time on-study)
Analysed for efficacy
(n=9)
On natalizumab (n=0)
(n=9)
Intention to treat analysis
(n=20)
12 completed 30 months of follow up (all-time on-study)
Analysed for efficacy
(n=12)
On natalizumab (n=2)
(n=10)
Intention to treat analysis
(n=20)

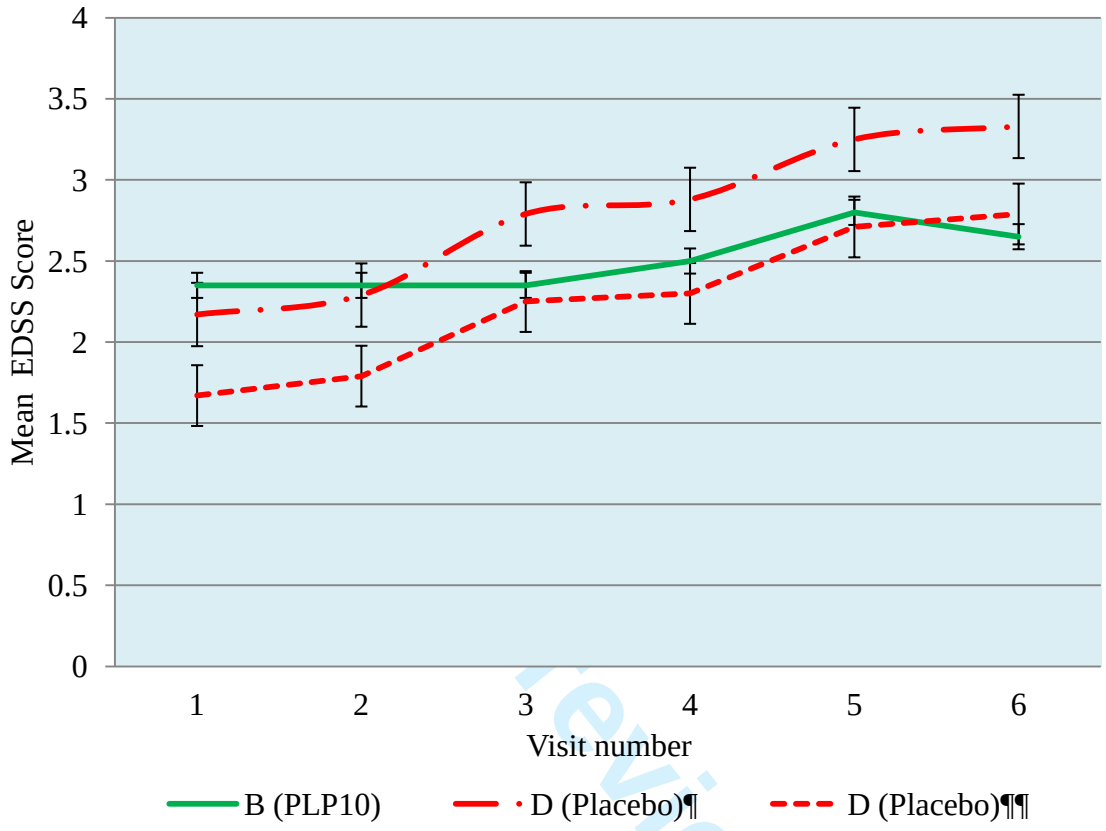


Possible effects on inflammation:

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


A
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Entry Baseline
0-6mo
0-12mo
0-18mo
0-24mo
PLP10 Group
Group A
Group C
Placebo Group
B
PLP10
Placebo
0.8
0.67
1
0
1
0.1
1.17
0.27
0-6mo
6-12mo
6-18mo
6-24mo
C
PLP10 group
Placebo group
1.35
0.83
0.4
0.83
0.4
1.04
Entry Baseline
1st year
Total 2-year
D
PLP10 group
Placebo group
Linear (PLP10 group)
Linear (Placebo group)
Number of Relapses
3.5
3
2.5
2
1.5
1
0.5
0
1 3 5 7 9 11 13 15 17 19 21 23
Month on Treatment


4A
Group
A
B (PLP10)
C
D (Placebo)
p=0.006 for PLP10 vs Placebo
Disability Progression
Confirmed after Six Months (%)
100
80
60
40
20
0
0 3 6 9 12 15 18 21 24 27 30
Time to First Progression (Month)
Number at risk
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
4B
Group
A
B (PLP10)
C
D (Placebo)
p=0.052 for PLP10 vs Placebo
Disability Progression
Confirmed after Six Months (%)
100
80
60
40
20
0
0 3 6 9 12 15 18 21 24 27 30
Time to First Progression (Month)
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



Supplementary Information

Table of Content

Supplementary Information Methods 1

Intervention and study rational. We are using high dosage of 1:1 omega-3:omega-6 polyunsaturated (PUFA) aiming to overpass and normalize global diet regional traditions and habits in relation to abnormal PUFA/saturated fatty acids (SFA)/monounsaturated fatty acids (MUFA) daily ratio consumption irrespective of the quantities consumed; and enough to equilibrate patients' diet in line with the recommended more physiologic omega-3:omega-6 ratio of about 1:1-4 wt/wt as reported by Simopoulos, 2002. In addition to correct existing deficiencies, cell membrane abnormalities, specifically of the immunopathological system and blood mononuclear peripheral cells, and high enough for availability and immediate ongoing modulation of the involved pathogenic mechanisms and network of events in MS. The high dosage is also required to overpass the quantity limitations, previously discussed, of diet-consumed PUFAs for cellular incorporation, especially in the central nervous system (CNS) of adults. Additionally, fatty acids (FAs) must first cross the intestinal epithelium before reaching the different tissues, where digestion and absorption constitute further problems in their availability (Carlier, H, 1991). Omega-3 PUFA are used in re-esterified form to eliminate unwanted disturbances, at the sides of action, by other fatty acids and molecules present in crude fish oils but also to increase the bioavailability of the FA since triglycerides have been shown to be associated with much higher bioavailability (Dyerberg et al, 2010). Linoleic (LA) and gamma linolenic acid (GLA) are essential structure molecules and important for any physiological (re)generation of cell membrane. GLA quantity is doubled to LA to ensure high direct production of dihomo-gamma-linolenic acid (DGLA), from GLA when LA cannot be metabolized, due to desaturase deficiency or malfunction. Such a reduced capacity to convert LA to GLA has been associated with aging, diabetes, alcoholism, atopic dermatitis, premenstrual syndrome, rheumatoid arthritis, cancer and cardiovascular diseases (Bolton-Smith et al, 1997; Horrobin, 1990; Leventhal et al, 1993). This is going to result in the increase of DGLA relative to arachidonic acid (AA) with DGLA promoting production of prostaglandin (PG)E1 but also inhibition of phospholipase (PL)A2: two major reasons and rational for their use. If other metabolic problems are involved within the omega-6 series and the normal metabolites are not produced then the eicosapentaenoic acid EPA available in PLP10 will substitute the function of DGLA, as a competitive inhibitor of AA for PLA2. In both cases the pro-inflamatory leucotrienes, prostaglandines of the 2-series (PG2) and thromboxanes of the 2 series including the platelet-activator factor (PAF) will be attenuated. The synthesis of AA from DGLA by Δ5 desaturase promoted by LA/GLA supplementation is very limited in humans as a result of limited activity of the enzyme (Yang-Yi & Robert, 1998). AA in the body is mostly available through diet. EPA and docosahexaenoic acid (DHA) are both physiologically important and crucial structured molecules able to substitute excess AA and SFA within the cell membranes. EPA will contribute to the inhibition (competitive to AA) of PLA2, joining the co-supplied omega-6 PUFA but will also participate in the production of anti-inflammatory leukotrienes, prostaglandins of the 3-series (PG3) and thromboxane (TX3) along with DHA, both found in the PLP10 intervention. Moreover EPA will replace AA of the membrane phospholipids and both omega-3 PUFA will contribute replacing abnormal quantities of SFA and excess AA. DHA is used in 3:1 ratio to EPA to cover any possible inabilities of EPA to be metabolized, high enough to strongly promote high production of the aforementioned anti-inflammatory

eicosanoids and cytokines and to be incorporated into CNS cell membranes where DHA should be the major PUFA present, replacing other FA, probably saturated and excess of AA. EPA, DHA, LA and GLA along with the rest of the other ingredients used ("other" omega-3 PUFA, SFA and MUFA that are usually found in the cell membranes of healthy people in limited quantities) in the intervention regimen are for their availability as minor structural constituents of physiological cellular membranes integrity, fluidity and overall function as building blocks for myelin repair and/or myelination. Furthermore the PUFA used within the cocktail intervention aimed to manipulate all other pathophysiological pathways that are reported to be able to: as previously discussed including gene transcription for neuroprotection and remyelination. Furthermore, PUFA are used to ensure the integrity of blood brain barrier (BBB) and to modulate the gelatinases responsible for the T cell migration within the CNS. Three different antioxidant vitamins (vitamin E, mostly as alpha-tocopherol, gamma (γ)-tocopherol (vitamin E isoform) and vitamin A) are used in the regimen preparation to support the cellular antioxidant defenses but also to protect peroxidation of the supplied increased amounts of PUFA. Alpha-tocopherol low-molecular-weight antioxidants will contribute to radical scavenging, interfering with gene transcription, protein expression, enzyme activity and metal chelation (van Meeteren et al, 2005). Vitamin E (alpha tocopherol) and vitamin A are used as antioxidants for the protection of the excess supplemented PUFA, with alpha-tocopherol been demonstrated to protect against peroxynitrite-induced oxidative damage, as well as able to efficiently detoxify hydroxyl, perhydroxyl and superoxide free radicals (the elevated reactive oxygen species (ROS)); each one with different mechanism of action, increasing the effect capability (Vatassery et al, 1998b; van Meeteren, 2005). Gamma-tocopherol is used in high dosage since its half life is very short compared to alpha-tocopherol and has been demonstrated to specifically protect against nitro-radicals. Tocopherols can also exert non-antioxidant properties, including modulation of cell signaling and immune function, regulation of transcription, and induction of apoptosis as previously discussed (van Meeteren et al, 2005).

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

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

adjunct to existing pharmaceuticals produced by reductionism, can be proven therapeutically superior to any available treatment for MS.

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Carlier H, Bernard A, Caselli C (1991) Digestion and absorption of polyunsaturated fatty acids. *Reproduction, nutrition, development* **31:** 475-500

Dyerberg J, Madsen P, Møller JM, Aardestrup I, Schmidt EB (2010) Bioavailability of marine n-3 fatty acid formulations. *Prostaglandins, leukotrienes, and essential fatty acids* **83:** 137-141

Horrobin DF (1990) Gamma-linolenic acid. *Rev Contemp Physiol* **1:** 1-41

Leventhal LJ, Boyce EG, Zurier RB (1993) Treatment of rheumatoid arthritis with gammalinolenic acid. *Annals of Internal Medicine* **119:** 867-873

Simopoulos AP (2002) The importance of the ratio of omega-6/omega-3 essential fatty acids. *Biomedecine & Pharmacotherapy* **56:** 365-379

van Meeteren ME, Teunissen CE, Dijkstra CD, van Tol EAF (2005) Antioxidants and polyunsaturated fatty acids in multiple sclerosis. *Eur J Clin Nutr* **59:** 1347-1361

Yang-Yi F, Robert SC (1998) Importance of Dietary g-Linolenic acid in human health and nutrition. *The Journal of Nutrition* **128:** 1411-1414

Vatassery GT, Smith WE, Quach HT (1998b) Alpha-tocopherol in rat brain subcellular fractions is oxidized rapidly during incubations with low concentrations of peroxynitrite. *Journal of Nutrition*128:152–157.

Supplementary Information Methods 2

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

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

The daily intervention formula agent dosages were:

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

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

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

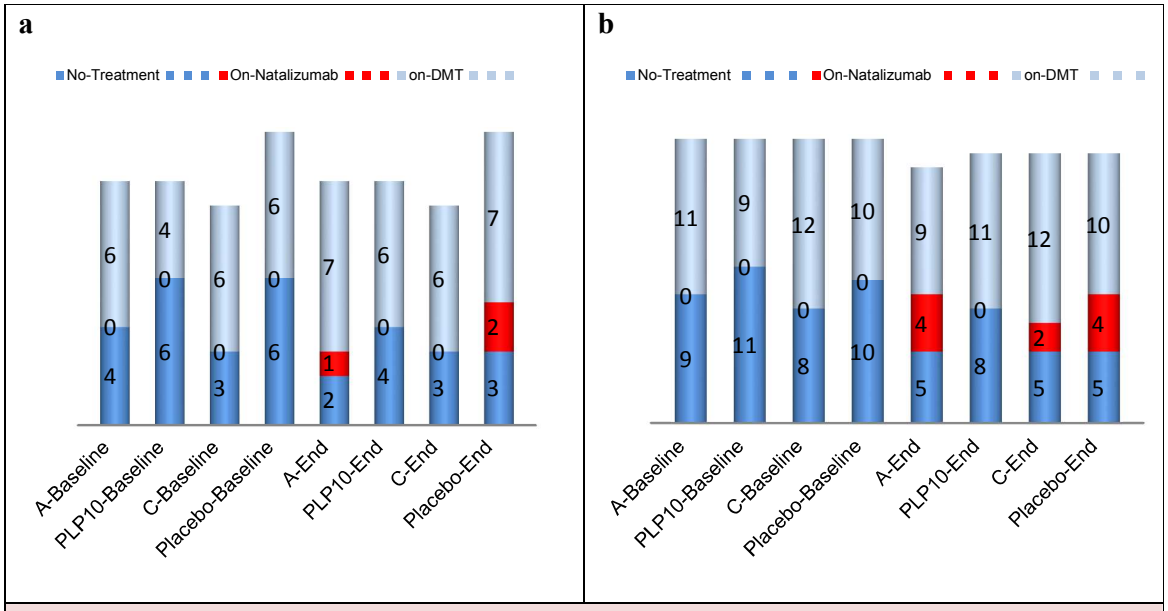
Intervention formula D daily dosage**:** pure virgin olive oil (16930mg).

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

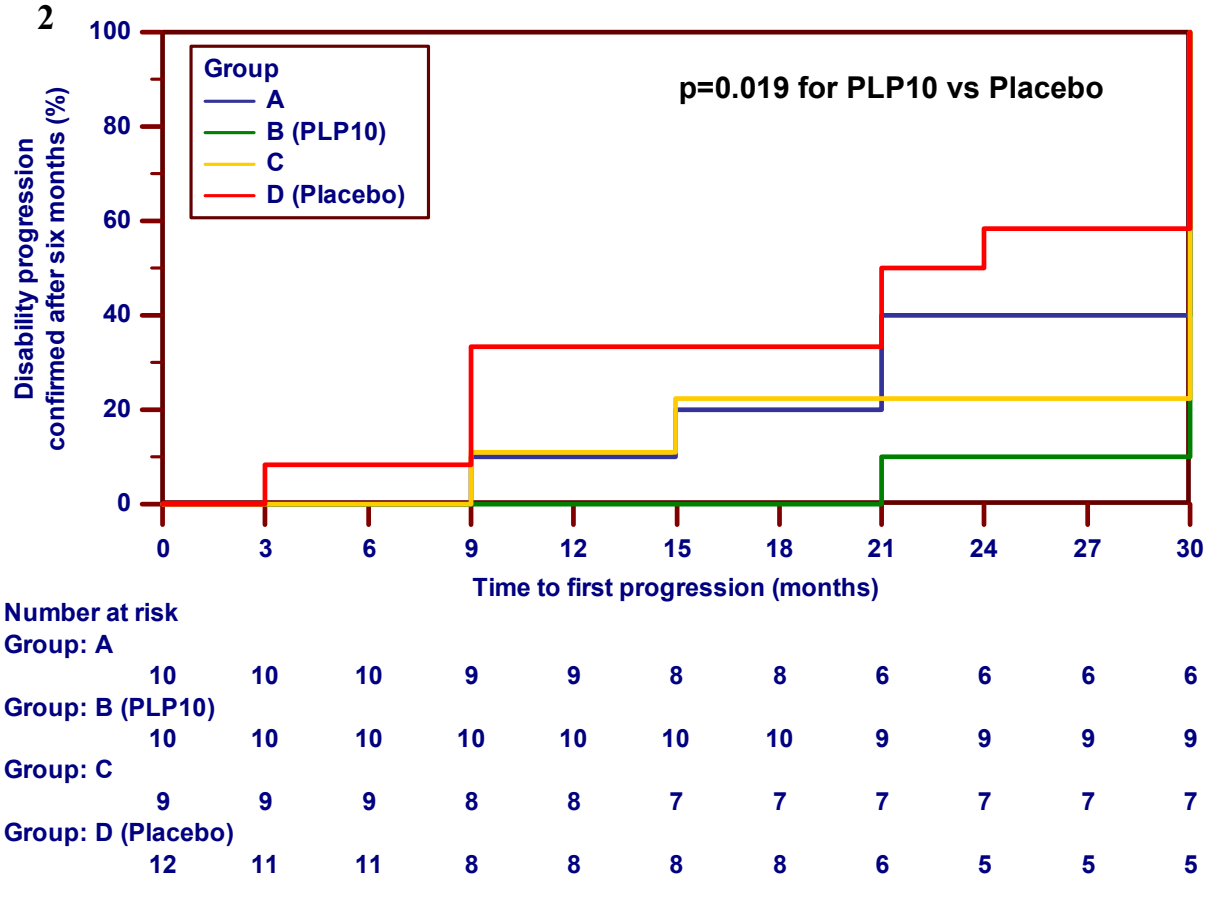
The specific omega-3 related fraction, according to specifications required for the interventions was prepared and purchased from EPAX AS, Aalesund, Norway; as re-esterified glycerides from fish body oils as a source. The specific omega-6 PUFA, MUFA and SFA related fraction, according to required specifications,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

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

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



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.



Supplementary Information Figure 2 | Kaplan–Meier estimates for the time to disability progression. Kaplan–Meier plot of the time to sustained progression of disability among all-time on-study patients, including patients on natalizumab, receiving intervention A, PLP10 and C *vs.* placebo. Intervention PLP10 reduced the risk of sustained progression of disability by 83% over two years (p=0.019). The cumulative probability of progression was 10% in the intervention B group and 58% in the placebo group. Intervention formula A reduced the risk of sustained progression of disability by 32% (p=0.301) and intervention formula C by 62% (p=0.109).

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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.

BMJ open

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:	*BMJ Open*
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

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

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Abstract

Objective To assess whether three novel interventions, formulated based on a systems medicine therapeutic concept, reduced disease activity in patients with relapsing remitting multiple sclerosis who were either treated or not with disease-modifying treatment.

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

Settings Cyprus Institute of Neurology and Genetics (CING)

Participants and Interventions Eighty subjects were randomised into four groups of twenty. 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 saturated fatty acids as well as vitamin A and vitamin E (alpha-tocopherol). The second intervention (B, PLP10) was a combination of A and γ-tocopherol. The third intervention (C) was γ-tocopherol alone. A fourth group of 20 received placebo. The interventions were administered per os (by mouth) once daily.

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

Results A total of 41 (51%) patients completed the 30-month trial. Overall, 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), representing 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). The per-protocol analysis for the secondary outcome at two years, the time to disability progression, was significantly longer only for 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, representing a relative 86% decrease in the risk of the 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). No adverse events were reported. Interventions A (10 patients) and C (9 patients) showed no significant efficacy.

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

Trial registration International Standard Randomised Controlled Trial, number ISRCTN87818535.

Introduction

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

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

The polyunsaturated fatty acid (PUFA) composition of membrane phospholipids plays an important role in immune- and non-immune-related inflammation. PUFA and antioxidant deficiencies along with a decreased cellular antioxidant defence mechanisms have been reported in MS patients.[12-15] The cause of PUFA deficiencies is not entirely clear and may involve metabolic and nutritional alterations.[12]

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

In vitro and *in vivo* studies have demonstrated that dietary eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), linoleic acid (LA), and gamma (γ)-linolenic acid (GLA) can be implicated and modulate almost all known complex networks of events and pathways in MS pathophysiology. The brain membrane fatty acid composition can be modified with dietary supplementation, but the process has been shown to be age dependent (taking much longer in adults versus developing brains) and possibly dependent on the quantity of the dietary/supplemented PUFAs.[18] Both human and animal studies proved that diets high in DHA and EPA can increase the proportion of these PUFAs in the membranes of

inflammatory cells and also reduce the levels of AA, a stress-related biomarker.[16 19] The anti-inflammatory properties of omega-3PUFAs include the production of PGs and TXs of the 3-series and LTs of the 5-series (Fig 2).[18 20] Resolvins and protectins are biosynthesised from omega-3 fatty acids via cyclooxygenase-2/lipoxygenase (COX-2/LOX) pathways, and they promote the control of inflammation in neural tissues (Fig 2).[21-25] T-cell proliferation in acute and chronic inflammation can also be reduced by supplementation with either omega-6 or omega-3 PUFAs.[26] Furthermore, vitamin E is an important antioxidant that can interrupt the propagation of free radical chain reactions.[27] Specifically, vitamin E (alpha-tocopherol, an isoform of vitamin E) efficiently detoxifies hydroxyl, perhydroxyl and superoxide free radicals, whereas γ-tocopherol (another isoform of vitamin E) appears to be more efficiently implicated in trapping NO radicals.[28 29] In addition, alpha–tocopherol exerts non-antioxidant properties, including the modulation of cell signalling and immune functions, regulation of transcription, and induction of apoptosis.[30]

Moreover, omega-3 fatty acid electrophilic derivatives formed by COX-2 in activated macrophages can stimulate the nuclear respiratory factor (Nrf2), which induces the transcription of neuroprotective and antioxidant-related genes and can activate the peroxisome proliferator-activated receptor (PPAR)γ for an anti-inflammatory response.[31-34] In animal studies, EPA and DHA proved to be endogenous ligands of RXRs, with positive effects on neurogenesis.[35] Additionally, in 2008, Salvati and coworkers reported evidence of accelerated myelination in DHA- and EPA-treated animals.[36] Moreover, DHA and EPA have been reported to significantly decrease the levels of metalloproteinases (MMP) -2, -3, -9, and -13, which have a significant role in the migration of lymphocytes into the central nervous system (CNS) by inducing the disruption of the blood brain barrier (BBB), an important step in the formation of MS lesions.[37-43]

Based on the above observations, specific PUFAs and antioxidant vitamins fulfil the criterion of biologic plausibility and have the potential to diminish the severity and activity of MS symptoms, potentially even promoting recovery (remyelination).[12 44]

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

Methods

Patients

The eligibility criteria were an age of 18 to 65; a diagnosis of RRMS according to the McDonald criteria; a score of 0.0 to 5.5 on the Expanded Disability Status Scale (EDSS), a rating that ranges from 0 to 10, with higher scores indicating more severe disability; MRI showing lesions consistent with MS; at least one documented clinical relapse; and either receiving or not a disease-modifying treatment (DMT) within the 24 month period before enrolment in the study. Patients were excluded because of a recent (<30 days) relapse, prior immunosuppressant or monoclonal antibody therapy, pregnancy or nursing, other severe disease compromising organ function, progressive MS, history of recent drug or alcohol abuse, use of any additional food supplements, vitamins, or any form of PUFA, and history of severe allergic or anaphylactic reactions or known specific nutritional hypersensitivity. No

monitoring or limitations on the patients' daily dietary habits were considered because the high quantities of the ingredients within the formula could not be significantly affected by any particular dietary pattern.

The study was conducted in accordance with the standards of the International Conference of Harmonisation Guidelines for Good Clinical Practice. The protocol was developed by the investigators, approved by the Cyprus National Bioethics Committee, and overseen by an independent safety-monitoring committee evaluating the safety and over-all benefit-risk profiles. The adherence of the care providers with the protocol was assessed by an external committee assigned by the funder of the project through reviews of case report forms. All patients gave written informed consent at the time of enrolment.

Randomisation and masking

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

The interventions had identical appearances and smells and were kept in dark bottles (15 daily-dose portions/bottle) under a nitrogen bed and labelled by HIONU with code numbers, blinded for both the patients and investigators. Study data were collected by the investigators

and saved by HIONU, which also held the blinded codes for the study. All study personnel involved in the conduct of the study were blinded throughout the study. The treating/examining physician, other investigators, pharmacist, neuroradiologist and patients were masked to the treatment allocation.

Procedures and end points

The specific omega-3 and omega-6 raw materials were purchased according to the required interventions' PUFA-fraction specification (molecular structure, quantity/ratio and quality) with vitamin E (alpha-tocopherol) used as antioxidant stabiliser by the supplier. The vitamins and masking aroma were purchased separately. The mixing of the fractions to the final required intervention-composition specification was always performed by the same team of scientists under the supervision of the involved medical biochemist and lipidology specialist and under appropriate conditions every six months. The interventions were refrigerated in the dark until use. See Table 1 and Supplementary Information Methods 1 and 2 for a detailed description of the interventions.

The participants were randomly assigned to receive the following: group A, a daily dose of a 19.5 ml mixture of 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 and 16:0 650 mg) / vitamin A (0.6 mg) / vitamin E (22 mg) plus citrus-aroma (intervention A); group B (PLP10), a daily dose of a 19.5 ml mixture of 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 and 16:0 650 mg) / vitamin A (0.6 mg) / vitamin E (22 mg) plus pure γ-tocopherol (760 mg) plus citrus-aroma (intervention B); group C, a daily dose of a 19.5 ml mixture of pure γ-tocopherol (760 mg) dispersed in pure virgin olive oil (16,137 mg) plus

citrus-aroma (intervention C); and group D (placebo), a daily dose of a 19.5 ml mixture of pure virgin olive oil (16,930 mg) plus citrus-aroma (intervention D) (Table 1). The pharmacist of the institution was responsible for the appropriate storage and handling of the interventions for the individual participants. The interventions were taken orally once daily 30 minutes before dinner using a dosage-calibrated cup for 30 months. The ingredients, ratio and dose were selected based on their biophysical interrelationship with the total known multiple MS causative factors, their biochemical importance and the role they were expected to play in the normalisation and treatment of the involved complex network of events in the disease pathophysiology. Moreover, the high intervention dosage was selected with the aim of optimising the body composition of omega-3 to omega-6 PUFAs to a 1:1 wt/wt ratio irrespective of dietary habits and geographical origin.

The period from July 1st 2007 (enrolment) to December 31st 2007 was used as the normalisation period. This six-month normalisation period would allow the interventions to exert their beneficial effect as oral PUFAs need four to six months to achieve pivotal action on immune and neural cells, correction of antioxidant deficiencies and body PUFA redistribution, and an optimal normalisation of the EPA and DHA ratio.[45-47] The study was completed on December 31st 2009 (30 months), and the recording of relapses continued until December 31st 2010 (42 months). Overall, the study included a "normalisation period" (July 1st 2007 to Dec 31st 2007), an "on treatment" period (Jan 1st 2008, the baseline, to Dec 31st 2009) and a 12-month "post-study monitoring period" (Jan 1st 2010 to Dec 31st 2010).

Depending on their clinical status and in accordance with common practice, the participants continued receiving their indicated regular treatment, with persistent evaluation for any side-effects and adverse events. Clinical assessment visits were scheduled at baseline and 3, 9, 15,

21 and 24 months on-treatment. The patients were also clinically examined by the treating neurologist within 48 hours after the onset of new or recurrent neurologic symptoms.

The primary end point was the ARR at two years. A relapse was defined as new or recurrent neurologic symptoms not associated with fever or infection that lasted for at least 24 hours and was accompanied by new neurologic signs. Relapses were treated with methyl-prednisolone at a dose of 1 g intravenous per day for three days, followed by prednisone orally at a dose of 1 mg/kg of weight per day on a tapering scheme for three weeks. The secondary end point at two years was the time to disability progression, defined as an increase of 1.0 or more on the EDSS and confirmed after six months. Progression could not be confirmed during a relapse, and the final EDSS score was confirmed six months after the end of the study. A post-hoc analysis was performed to assess the proportion of patients free from new or enlarging T2 lesions on brain MRI scans at the end of the study for the per-protocol participants of the group receiving the most effective intervention versus placebo. This comparison was made versus the available archival MRI scans up to three months before the enrolment date. The MRI scans were performed and blindly analysed at an MRI evaluation centre. The patients were monitored for an additional 12 months after completion of the trial, and relapses were recorded. The patients were strongly encouraged to remain in the study for follow-up assessments even if they had discontinued the study drug.

Blood samples were collected from all randomised patients at the time of enrolment, at every scheduled clinical assessment and during relapses. To evaluate the compliance, the fatty acid composition of the patients' red blood cell membranes was determined by gas chromatography, according to a standard protocol. The fatty acid analyses were performed after study termination and thus did not influence the blinding. Safety measures were

assessed from the time of enrolment until 12 months following the study completion. Haematological and biochemical tests were performed at enrolment and every 12 months, including a full blood count, renal and liver function tests, and protein, cholesterol, triglyceride, glucose and electrolyte levels.

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

The whole procedure followed the clinical trial guidelines as required by the USA Food and Drug Administration, European Medicines Agency, and the Committee for Medicinal Products for Human Use.[48]

Statistical analysis

Power calculations could not be performed before the study because of the lack of information from previous studies on the potential effect sizes. In 2005, the prevalence of MS in Cyprus (600,000 population) was 120/100,000. Based on the aforementioned MS patient numbers in our country and the reference centre, the CING, we were able to enrol 20% of the

total RRMS patients eligible for treatment. The sample size was strictly based on the subjects' availability and the novelty of the assessed intervention.

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

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

For the secondary end-point, the time to disability progression, Kaplan–Meier curves were constructed. The progression of disability and time thereof were compared in a pair-wise fashion for the active interventions versus placebo by the log-rank test in the main analysis and by the Cox proportional-hazards models with adjustments for the baseline EDSS score, age and DMT in the supportive analysis. Multivariate models considered all variables with P <0.1 in the univariate models. There was no overt violation of the proportionality assumption.

Both per-protocol and intention to treat (ITT) analyses were performed for different sets of research questions to be answered, and both are reported. Missing data of the five patients lost to follow-up were imputed by the last-observation-carried-forward (LOCF) approach. Due to the proof-of-concept design of the study, the considerable non-adherence rate (49%)

and the resulting interpretation issues regarding the ITT analysis, the per-protocol analysis was considered to be the more informative and appropriate method to answer the research questions addressing the efficacy of the interventions when the subjects continuously followed the protocol. All statistical analyses were well defined a priori. All analyses were performed with STATA SE 10.0 (College Station, TX, USA). P-values were two-tailed.

Role of the funding source

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

Results

Study population

Among the 80 patients, 20 patients were randomly assigned to each of the three groups to receive the interventions, and 20 to receive the placebo (Fig 1). The baseline characteristics of both the ITT and per-protocol populations were similar across the groups (Table 2A and 2B). All patients who dropped out completed the follow-up until the study completion and were included in the ITT analyses (Table 4). Five patients were lost to follow-up before their first scheduled visit. Two other patients who dropped out before their first scheduled visit progressed to secondary progressive MS. Fifteen patients dropped out without successfully completing the “normalisation” period, including five pregnancies. Another 17 patients dropped out early after the entry baseline. Seven patients who dropped out were given

monoclonal antibody treatment (natalizumab). Overall, a total of 41 (51%) patients completed the 42-month study, one patient from group A and two from the placebo group transferred to natalizumab, and 39 (49%) patients either withdrew (dropped out) or were lost to follow-up. The reasons for discontinuation are listed in Figure 2.

Efficacy

Relapses

As a proof-of-concept trial, we primarily needed to answer whether the interventions were effective for those patients who adhered to the assigned treatment, which was the per-protocol analysis.[49] For methodological comprehensiveness, we also performed the ITT analysis as a secondary analysis to answer different questions that were complementary to our core hypothesis, such as what happened to all MS patients who were placed on the interventions (the effect of assignment).[49]

In the per-protocol analysis, during the first year of the treatment, the ARR was 0.80, 0.40, 0.78 and 0.83 for the four intervention groups, respectively. During the second year, the ARR was 0.90, 0.40, 0.67 and 1.25 for the four intervention groups, respectively. Overall, for the two-year primary end point, 8 relapses were recorded for the 10 patients in the PLP10 group (0.40 ARR) versus 25 relapses for the 12 patients on the placebo (1.04 ARR), a 64% adjusted relative rate reduction (RRR) for the PLP10 group (RRR 0.36, 95% confidence interval [CI] 0.15 to 0.87, p=0.024) (Tables 3A, 5 and Fig 3A and 3C). After excluding 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, Tables 3B and 5). Pair-wise comparisons for the other two groups against the placebo did not yield significant results (Tables 3A, 3B). The proportion of patients with ≤1 relapse for the two years on-study was

higher in the PLP10 group than in the placebo group (90% versus 42%, p=0.030, Table 5). Seeking to further investigate the observed difference, we compared the relapse rate during the 24 months before the entry into the study to the 24 months on-treatment for each intervention group. We observed a significant relative reduction in the ARR (70%) only in the PLP10 group (RRR 0.30; 95% CI 0.14 to 0.65, p=0.003, Table 3A); within-group comparisons for the ARR reduction of the three other groups were not significant and remained not significant when the natalizumab-treated patients were further excluded from the analysis. The effect of PLP10 through time at different time-windows versus placebo for all-time on-study patients is shown in Figures 3A to 3D. Although the ARR analysis within time-windows was not an assigned endpoint, it could help with the process of evaluating parallel information, such as the efficacy profile through time. PLP10 reached its maximum effect within one year on-treatment (counted from the entry baseline) and remained stable afterwards at an ARR of 0.4 with some free-relapse time-windows. Figure 3D demonstrates the dispersion of relapses throughout the 2-year period of all-time on-study (excluding patients on natalizumab) for PLP10 (n=10) versus placebo (n=10). The placebo group, in line with the existing knowledge of how the relapse history works in relation to future relapses in MS patients (contagion phenomenon), showed the expected trend of increased relapse incidences.[50] The same phenomenon was true for groups A and C. Finally, during the 12 month post-study extended period, the on-study patients who received PLP10 showed a persistent benefit in the ARR compared with the placebo (six relapses for the 10 subjects within the PLP10 group, 0.6 ARR versus 19 for the 12 subjects within the placebo group, 1.58 ARR), indicating a statistically significant 62% adjusted relative rate reduction in the ARR for the PLP10 group (RRR 0.38, 95% CI 0.12 to 0.99, p=0.046).

Regarding the ITT analysis, 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 (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 with the 24 months on-treatment for the PLP10 group (RRR 0.45, 95% CI 0.26 to 0.78, p=0.005).

Disability progression

In the per-protocol analysis, at two years, the time to disability progression 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 the patients on natalizumab, there was again a statistically significant difference between the PLP10 and 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 86% decrease in the risk of the sustained progression of disability 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 placebo groups, respectively, when patients on natalizumab were excluded. No statistically significant difference was observed for any comparison of the other two groups with the placebo group (Fig 4A and Supplementary Information Fig 2).

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

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

MRI

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

Safety

Over the course of the 30 month study, no significant adverse events were reported for any group. The only aetiology for the drop-outs was the palatability and smell of the formula preparations in addition to pregnancy. Nausea was reported by two patients. No abnormal values were observed on any of the biochemical and haematological blood tests. No allergic reactions were reported.

Discussion

In this proof-of-concept, randomised, double-blind clinical trial assessing the safety and efficacy of three variations of a novel nutritional formula in RRMS, we observed a significant association for a formula containing a balanced mixture of specific omega-3 and omega-6 PUFAs, MUFAs, SFAs, vitamin A, vitamin E and γ-tocopherol (PLP10) compared with the placebo for both the ARR and the progression of disability in the per-protocol analysis. Our results included analyses pertaining to a total of 42 months of study-collected data, including the 12-month intervention-free treatment extension period. We also observed a high drop-out rate that was mostly the result of formula palatability, a common phenomenon in trials using oily interventions. Interestingly, a statistically significant reduction in the ARR and disability progression was also observed when comparing the ARR of the PLP10 patients in the 24-month period prior to the study with the ARR of the 24 months on-study; the observed differences became larger when the patients who received natalizumab (the currently most potent disease modifier) were excluded. The ARR decreased within a year on PLP10 and remained stable until the study completion. The statistically significant difference in the ARR between patients on PLP10 and those on placebo continued for the 12 month extended period (persistent effect) without a significant difference on the DMT. These clinical findings are supported by the results from the MRI analysis, in which the proportion of patients free from new or enlarging brain T2 lesions was also higher in the PLP10 group than the placebo group. No severe side effects have been reported.

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

is well known that the majority of the patients suffering from MS do use dietary supplements for a variable length of time.[52] Dietary antioxidants and fatty acids may influence the disease process in MS by reducing immune-mediated inflammation, oxidative stress and excitotoxic damage.[12] Published data have revealed that healthy dietary molecules have a pleiotropic role and are able to change cell metabolism and down-regulate inflammation by interacting with enzymes, nuclear receptors and transcriptional factors.[51] Current available treatments are the products of reductionism, partially effective and associated with severe side effects. Interferons and glatiramer acetate, the most widely used first-line MS drugs available today, are associated with the least severe side effects among the MS therapies, but they are reported to reduce the ARR only by about one third and with no significant effect on the progression of disability.[53] Natalizumab reduces the ARR by 68% and decreases the possibility of disability progression by 43%, with 57% of patients free of new or enlarging T2 lesions on MRI scans, compared with 15% on placebo.[54] Fingolimod is associated with a 54% ARR reduction (without a significant benefit on the progression of disability). Both natalizumab and fingolimod are second-line drugs associated with severe side-effects.[55]

Mehta in a review paper, in 2009, reported different clinical studies on interventions formulated based on the individual aforementioned molecular ingredients or based on a specific ratio of the aforementioned molecular ingredients for MS treatment; although no one was reported using the antioxidant vitamin γ-tocopherol.[56] In our study, the choice of ingredient proportion and dosing scheme was based upon evidence derived form *in vivo* and *in vitro* data. In the Western diet, the ratio of omega-3 to omega-6 is approximately 1:20–30; in populations that consume fish-based diets, the ratio is approximately 1:1–2.[57 58] The intervention daily dose was aiming to be, and believed to be, high enough to restore/amplify body-efficient antioxidant activity and ensure cellular membranes lipid profile normalisation

(PUFA content) and simultaneously potentiate the involvement of the ingredients in the anti-inflammatory and recovery mechanisms. Dietary fatty acid molecules need an approximately six month period to exert their beneficial effect, and this essential parameter was under consideration for the first time in our study design (normalisation period).[46] This chronotherapy parameter might be of major importance and is in line with the systems medicine treatment philosophy. We believe that the persistent effect within the post-study period is in agreement with the reported very long washout phase for omega-3 fatty acids, especially DHA, to return to the pre-treatment values.[46] Considering that omega-3 PUFA supplementation can promote the replacement of AA within the cellular membranes, we can speculate that an increased inflammatory activity can possibly result during the first six months of supplementation.

In addition to EPA, DHA, LA, and GLA, PLP10 contained limited quantities of other structural/active PUFAs, specific MUFAs (mostly oleic acid) and SFAs (palmitic and stearic acids), specifically to provide a direct source for neuronal cell membrane rehabilitation and for (re)myelination and neuroprotection because these compounds are all major components, precursors and building blocks of any new physiological myelin and cellular membranes in general. Assembly of the correct molecules into the myelin membrane may be especially critical during active synthesis. If these critical constituents are not directly or indirectly available, amyelination, dysmyelination or demyelination may ensue.[59] The maintenance of myelin requires continued turnover of its components throughout life.[60 61]

Different factors and molecular entities appear to be part of the possible aetiology for MS, with specific PUFAs and antioxidants found to be key substances related to all known pathogenic and recovery mechanisms. In our study, we further propose that a holistic systems

medicine model approach can be applied by synchronised action. First, there is an obvious convenience in administering one formula containing different specific active ingredients. The currently available evidence supports that nutritional interventions would confer a small to medium treatment effect with an accompanying appropriate safety profile.[12 52 56] Combining these specific active ingredients together with γ-tocopherol and other specific active molecules into one stable formulation is expected to enhance adherence while still offering an appropriate safety profile. A similar approach could not be adopted for pharmaceutical interventions with common and severe adverse events, such as those indicated today for patients with MS. Given the advantages of the simultaneous use and that all the included ingredients have proven individually a valid biological plausibility and have been tested in various settings and under various dose schemes, we also assessed the hypothesis that a novel mixture of these ingredients would have a postulated efficacy attained synergistically through different mechanisms of action.[52 56] Interestingly, the observed magnitude of the treatment effect cannot be explained by adding up the postulated efficacy estimates of the individual ingredients. Findings from *in vitro* and *in vivo* studies support this notion of proposed synergy although this hypothesis can only be taken forward when the observed treatment effect is validated in various settings and in a larger number of patients.

We acknowledge that our study has two considerable limitations: the small sample size and the high drop-out rate. Regarding the sample size, one should bear in mind that this study is a small, phase-2 clinical trial assessing a novel intervention and thus has comparable size in the appropriate literature. Questions taken forward from this trial can be assessed in a larger randomised trial in which appropriate power calculations would be possible, taking into consideration the findings of the present study. The adherence of the subjects is another limitation of our study, but the total duration of the study that covers a total of 42 months

follow-up adds power to the results.[48] We acknowledge that we had to deliver the intervention in the way most frequently associated with low compliance, i.e., an oral, liquid formula, thus triggering maximum intolerance due to taste. Nevertheless, the observed suboptimal compliance is in accordance with the published literature in which clinical trials assessing liquid fatty acid interventions show a weaker adherence compared with clinical trials of pharmaceutical interventions. Indeed, in our study, we consistently recorded the reasons for withdrawal: most of the participants did not discontinue due to safety issues, but rather due to palatability issues. Controlling non-compliance due to palatability issues is by far easier to address compared with non-compliance related to adverse events and can be resolved when optimisation of the formulation is achieved in future trials. At this stage of the development of the intervention, we would by far exceed the cost-effectiveness threshold if we were to invest in improving these features of the intervention. Moreover, we should also note that MS patients are subject to far more frequent and more serious adverse events related to the current standard treatments.

As a direct consequence of the low compliance and the loss of power, the performed intention-to-treat analysis was far less robust than intended, and we would then have to take into serious consideration the performed per-protocol analysis. We focused on the per-protocol data analysis because it is the appropriate method to best provide the answer for the proof-of-concept trial-addressed question.[24] To validly incorporate the results of the per-protocol analysis into the interpretation of the overall results of the trial, we needed to ensure that the randomisation was not seriously violated due to the exclusion of the non-compliers. The comparison between the baseline characteristics of the patients included in the per-protocol analysis did show a relative balance in the compared groups for known confounders. Nevertheless, the presence of unknown confounders introducing bias to the trial results

cannot be excluded despite non- significant differences in the baseline characteristics. As an additional safeguard towards that end, we also performed adjusted analyses for the primary and secondary analyses for important clinical and demographic parameters, i.e., relapses, EDSS, age and DMT.

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

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.

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.

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-

treatment, the ARR of group A was 0.85, with an 18% decrease compared with placebo (p=0.468); that 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% decrease (p=0.578). This section also reports the comparison of the 24 mo pre-treatment ARR (baseline ARR) with the 24 mo on-treatment ARR of the all-time on-study population, including patients on natalizumab.

Section 3B reports the comparison of the 24 mo pre-treatment ARR with the 24 mo on-treatment ARR of the all-time on-study population excluding patients on natalizumab and the comparison of the ARR during the 24 mo period on-treatment (primary end point) for each treatment group compared with the placebo.

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

baseline and decreased to 13 during the two-year study period. These results are expected because for the 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 the 57% of the placebo group drop-outs who were under DMT at entry baseline increased to 86% at the end of the study, including two patients on natalizumab.

Section 4B reports the comparison of the 24 month pre-treatment ARR (baseline) with the 24 month on-treatment ARR for the total randomised population by treatment arm. The ARR of the PLP10 group was 1.23 at baseline and 0.75 at the end of the study (39% reduction, p=0.005), and that for the placebo group was 1.08 at baseline and 1.03 at the end of the study (5% reduction, p=0.652). No significant difference was calculated for the other two treatment arms. During the 24 months on-treatment, the PLP10 group presented a 27% reduction in the ARR versus the placebo group (p=0.121), with all groups lacking statistically significant results.

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					
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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Ethical approval: Cyprus National bioethics committee (reference ΕΕΒΚ/ΕΠ/2005/10).

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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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Figure legends

Figure 1. Study Flowchart

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

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

Abbreviations: PL, phospholipid; IFN-γ, interferon γ; IL-2, interleukin 2; NFκB, nuclear factor kappa B; PGE2, prostaglandin E2; PPARγ, peroxisome proliferator-activated receptor γ; PUFAs, polyunsaturated fatty acids; TGFβ, transforming growth factor β; TNF, tumour 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.

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

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

Panel C demonstrates the ARR of the all-time on-study population for the PLP10 *vs.* placebo groups at baseline, during the 1st year, and during the 2nd year on-treatment. **

Panel D demonstrates the dispersion of relapses throughout the 2-year period of all-time on-study (excluding patients on natalizumab) for PLP10 (n=10) *vs.* placebo (n=10). The placebo group showed an irregular dispersion of relapses compared with the PLP10 group, with a linear increasing trend, whereas the PLP10 group showed a stabilised linear trend. Using the per-protocol model in which the patients on natalizumab were excluded, the number of relapses could be compared on the same number of patients.

** Including the patients on natalizumab.

Figure 4. Panel 4A demonstrates the Kaplan–Meier plot of the time to sustained progression of disability among the all-time on-study patients, excluding the patients on natalizumab, receiving interventions A, PLP10 and C compared with placebo. PLP10 reduced the risk of the sustained progression of disability by 86% over two years (p=0.006). Intervention formula A reduced the risk of the sustained progression of disability by 53% (p=0.266), and intervention formula C, by 67% (p=0.061).

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

Figure 5. Mean change in the expanded disability status scale score as a function of visit number. The values are expressed as the mean ± standard error of the mean (s.e.m.)

¶ Including patients on natalizumab

¶¶ Excluding patients on natalizumab

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

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Keywords: antioxidant vitamins, polyunsaturated fatty acids, multiple sclerosis, systems medicine, ~~randomized~~ randomised clinical trial.

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Abstract

Objective To assess whether ~~our~~ three novel interventions, formulated based on systems medicine therapeutic concept reduced disease activity in patients with relapsing remitting multiple sclerosis who were either treated or not with ~~disease~~ disease-modifying treatment ~~or untreated~~.

Design A 30-month ~~randomized~~ randomised 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~~ Eighty subjects were ~~randomized~~ randomised into four groups of ~~20~~twenty. 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 (by mouth) once daily.

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

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Results A total of 41 (51%) patients completed the 30-month trial. Overall, 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), representing 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). The ~~P~~per-protocol analysis for the secondary outcome at two years, the time to disability progression, was significantly longer only ~~with~~ for 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, representing a relative 86% decrease in the risk of the 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). No adverse events were reported. Interventions A (10 patients) and C (9 patients) showed no significant efficacy.

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

Trial registration International Standard ~~Randomized~~ Randomised Controlled Trial, number ISRCTN87818535.

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Introduction

Multiple sclerosis (MS) is a complex ~~multifactorial~~ disease that results from the interplay between ~~as yet unidentified~~ environmental factors and a ~~disease~~ susceptible genetic background~~susceptibility genes~~.[1-~~4~~3] Together, these factors trigger a cascade of events, involving the engagement of the immune system, inflammatory injury of myelin, axons and glia, functional recovery and structural repair, gliosis, and neurodegeneration.[4] The ~~bio~~ mechanisms involved ~~are~~include~~.~~ immune-mediated inflammation, oxidative stress and excitotoxicity,~~.~~[5-~~9~~10] ~~and they These mechanisms may all~~all of which contribute to oligodendrocyte and neuronal damage and even cell death, hence promoting disease progression. The increasing prevalence of MS~~, limited varying~~combined with the partial efficacy and ~~the~~ side effects of the existing treatments have urged the ~~clinical need for the~~ development of new, innovative, more effective, safe,~~ –~~ and preventive treatment strategies ~~(ref?).~~

Recent ~~R~~research has shown that multiple variables dynamically interact and many different complex interrelated processes are simultaneously orchestrated for MS pathogenesis. The ~~fundamental~~ uniqueness ~~distinctiveness~~ of systems medicine (SM) is ~~not just~~ the recognition that different specific complex factors are important in disease management~~, but~~ and that ~~they~~ these factors need to be incorporated in some meaningful way ~~to~~ for treatment selection and delivery.[10] The primary challenge ~~tackled by~~of a systems scientific approach is the elucidation of how these multiple variables dynamically interact and how ~~one can apply~~ this understanding can be applied to affect the system and achieve a desirable end.[10] One approach towards that end ~~The answer~~ might be the simultaneous ~~interference~~ intervention ~~with~~ in multiple~~all~~ involved ~~perturbed mechanisms~~pathways, ~~by~~ using a ~~cocktail~~ combination of different ~~specific~~ active ingredients ~~which~~that could exert a ~~, potentially able through~~

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

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

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

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

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

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Moreover, omega-3 fatty acids electrophilic derivatives formed by COX-2, in activated macrophages can stimulate the nuclear respiratory factor (Nrf2), which inducing induces the transcription of neuroprotective and antioxidant–related genes, and can activate the peroxisome proliferator-activated receptor (PPAR)γ for an anti-inflammatory response.[27-29] In animal studies, EPA and DHA proved to be endogenous ligands of RXRs, with positive effects on neurogenesis.[30] Additionaly, in 2008, Salvati and coworkers reported evidense evidence of accelerated myelination in DHA- and EPA-treated animals.[32] Moreover, DHA and EPA are have been reported to significantly decrease the levels of metalloproteinases (MMP) -2, -3, -9, and -13, with which have a significant role in the migration of lymphocytes into the central nervous system (CNS) by inducing the disruption of the blood brain barrier (BBB), an important step in the formation of MS lesions.[33-39]

Based on the above observations, specific PUFA and antioxidant vitamins fulfill the criterion of biologic plausibility and have the potential to diminish the severity and activity of MS symptoms severity and activity, potentially even promoting recovery (remyelination).[11] Overall, PLP10 contains multiple ingredients (omega-3, omega-6 and other fatty acids and vitamins) potentially able to modulate key interconnected components (i.e. genes, proteins) and structural molecules (i.e. cellular membrane lipids, receptors) within the functional network of events of MS pathogenesis.[40]

We report here This is a randomized randomised phase II, single-center, double-blind, placebo-controlled, proof-of-concept clinical trial evaluating the therapeutic ability of a nutraceutical formula (with PLP10 representing the complete composition of the formulation) and of two other interventions (A and C) consisting of PLP10-constituent partial fractions

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containing ingredients for the aforementioned substance categories on relapsing remitting (RR) MS patients.

Methods

Patients

The eEligibility criteria were an age of 18 to 65; a diagnosis of RRMS according to the McDonald criteria; a score of 0.0 to 5.5 on the Expanded Disability Status Scale (EDSS), a rating that ranges from 0 to 10, with higher scores indicating more severe disability; MRI showing lesions consistent with MS; and at least one documented clinical relapse; and either receiving or not a disease modifying treatment (DMT) within the 24 months period before beginning (enrollment) inof the study. Patients were excluded because of a recent (<30 days) relapse, prior immunosuppressant or monoclonal antibodies antibody therapy, pregnancy or nursing, other severe disease compromising organ function, progressive MS, history of recent drug or alcohol abuse, use of any additional food supplement, vitamins, or any form of PUFA, and history of severe allergic or anaphylactic reactions or known specific nutritional hypersensitivity. No monitor or limitations on the patients' daily dieryt habits were included consideredin the study design since because the high quantities of the ingredients within the formula s daily dosage ecould not be significantly affected or spoiled by any particular dietary pattern by any confounding factors within any known global daily food diet (see procedures, treatment regimen and end points).

The study was conducted in accordance with the standards of the International Conference of Harmonization Guidelines for Good Clinical Practice. The protocol was developed by the investigators and it was approved by the Cyprus National Bioethics Committee and was

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overseen by an independent safety-monitoring committee evaluating the safety and overr~~r~~all benefit-risk profiles. The adherence of care providers with the protocol was assessed by an external committee assigned by the funder of the project through reviews of case report forms. All patients gave written informed consent at the time of enrolment.

~~Randomization~~ Randomisation and masking

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

The interventions had identical appearance and smell and were kept in dark bottles (15 daily-dose portions/bottle) under nitrogen bed and labeled by HIONU with code numbers, ~~unidentifiable~~ blinded for both patients and investigators. Study data were collected by the investigators and saved by the HIONU, which ~~that~~ also held the blinded codes of the study. All study personnel involved in the conduct of the study were blinded throughout the study. The ~~T~~treating/examining physician, ~~all~~ other investigators, ~~the~~ pharmacist, ~~the~~ neuroradiologist and ~~all~~ patients were masked to treatment allocation.

Procedures and end points

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The specific omega-3 ~~(re-esterified glycerides)~~ and omega-6 ~~(glycerides)~~ raw materials were purchased according to the required interventions' PUFA-fraction specification (molecular structure, quantity/ratio and quality) with vitamin E (alpha-tocopherol) used as antioxidant ~~stabilizer~~ stabiliser by the supplier. The vitamins and masking aroma were purchased separately. The mixing of fractions to the final required intervention-composition specification was always performed by the same team of scientists under the supervision of the involved medical biochemist and lipidology specialist and~~,~~ under appropriate conditions every six months. The ~~I~~interventions were ~~stored~~ refrigerated in the dark until use. See Table 1 and Supplementary Information Methods 1 and 2 for ~~the~~ a detailed description of the interventions ~~specification detailed description, and study/intervention rational.~~

The ~~P~~participants were randomly assigned to receive the following: ~~in~~ group A, a daily dose of a 19.5ml mixture of 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 A); ~~in~~ group B PLP10, a daily dose of a 19.5ml mixture of 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 pure γ-tocopherol (760mg) plus citrus-aroma (intervention B); ~~in~~ group C, a daily dose of a 19.5ml mixture of pure γ-tocopherol (760mg) dispersed in pure virgin olive oil (16,137 mg) plus citrus-aroma (intervention C); and ~~in~~ group D (placebo), a daily dose of a 19.5ml mixture of pure virgin olive oil (16,930mg) plus citrus-aroma (intervention D) (Table 1). The ~~institution's~~ pharmacist of the institution was responsible for the appropriate storage and handling of the interventions ~~to~~ for the individual participants. The interventions were taken orally once daily 30 minutes before dinner by using a dosage calibrated cup for 30 months.

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The ingredients, ratio and dose ~~have been~~were selected based on their biophysical interrelation ~~to~~ with the total known multiple MS ~~causing~~ causative factors, their biochemical importance and the role they were expected to play in the normalisation and treatment of the involved complex network of events in the disease pathophysiology. Moreover, the high intervention ~~intake~~ dosage was selected with the aim of optimising the body composition of omega-3 to omega-6 PUFAs to a 1:1 wt/wt ratio irrespective of dietary habits and geographical origin.

~~used to overcome any abnormal dietary accumulation of related agents as a result of patients' food intake habits, irrespective of geographical origin, in relation to the daily consumption ratio of the total fatty acid intake; in order to end up with omega-3 to omega-6 PUFA indicated physiological body ratio composition of 1:1 wt/wt.~~

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

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Depending on their clinical status and in accordance with the common practice~~the ethical issues governing clinical trials~~, the participants continued receiving their ~~indicative~~ indicated regular treatment~~.~~ , with persistent evaluation for any side-effects and adverse events. Clinical ~~assessments~~ assessmen visits were scheduled at ~~entry~~ baseline~~,~~ and 3, 9, 15, 21 and 24 months on-treatment. The ~~P~~patients were also clinically examined by the treating neurologist within 48 hours after the onset of new or recurrent neurologic symptoms.

The primary end point was the ~~annualized relapse rate (~~ARR~~)~~ at two years. A relapse was defined as new or recurrent neurologic symptoms not associated with fever or infection that lasted for at least 24 hours and was accompanied by new neurologic signs. Relapses were treated with methyl-prednisolone at a dose of 1g intravenous per day~~,~~ for three days followed by prednisone orally at a dose of 1mg/kg of weight per day on a tapering scheme for three weeks. The secondary end point at two years was the time to ~~confirmed~~ disability progression, defined as an increase of 1.0 or more on the EDSS and confirmed after six months. ~~(p~~Progression could not be confirmed during a relapse~~).~~ and t~~T~~he final EDSS score was confirmed six months after the end of the study. A post-hoc analysis was performed to assess ~~ing~~ the proportion of patients free from new or enlarging T2 lesions on brain MRI scans at the end of the study for the per-protocol participants of the group receiving the ~~highest~~ most effective intervention versus placebo. This ~~C~~comparison was made versus the available archival MRI scans up to three months before the enrolment date. The MRI scans were performed and blindly analyzed at an MRI evaluation centre. The patients ~~continued to be followed~~were monitored for an additional 12 months after completion of the trial and relapses were recorded. ~~Finally,~~The patients were strongly encouraged to remain in the study for follow-up assessments even if they had discontinued the study drug.

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Blood samples were collected from all ~~randomized~~ randomised patients at the time of enrolment, at every scheduled clinical assessment and during relapses. To ~~check~~ evaluate the ~~individual~~ compliance ~~with intake~~, the fatty acids composition of the patients' red blood cell~~s~~' membranes was determined~~,~~ by gas chromatography, according to a standard protocol. The fatty acid analyses were performed after study termination and thus did not influence the blinding. Safety measures were assessed from the time of enrollment until 12 months following the study completion. Haematological and biochemical tests were performed at enrolment and every 12 months, including full blood count, renal and liver function tests, and proteins, cholesterol, triglycerides, glucose and electrolyte levels.

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

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

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Statistical analysis

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

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

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

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

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

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

Role of the funding source

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

Results

Study population

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

Among the 80 patients, 20 patients were randomly assigned to each of the three groups to receive the interventions, and 20 to receive placebo (Fig 1). The ~~B~~ baseline characteristics of both the ITT and the per-protocol populations were similar across groups (Table 2A and 2B). All patients ~~that~~ who droped ~~-~~out ~~had a~~ completed the~~d~~ follow-up until the study completion and were included in the ITT analyses (Table 4). Five patients were lost to follow-up before their first scheduled visit. T~~and t~~wo other patients who ~~that~~ dropped-out before their first scheduled visit progressed to secondary progressive MS. Fifteen patients dropped ~~-~~out without successfully completing the "normalization" period, ~~-~~including five pregnancies. Another 17 patients dropped-out early after the entry baseline. Seven patients ~~that~~ who dropped out were given monoclonal antibody treatment (natalizumab). Overall, a total of 41 (51%) patients completed the 42-month study ~~(July 2007 through December 31st 2010, including the 12-month extended period),~~ ~~where~~ one patient from group A and two from the placebo group transferred on natalizumab, and 39 (49%) patients either withdrew (dropp~~-~~ed out) or lost to follow. The ~~R~~reasons for ~~study interventions~~ discontinuation are listed in Figure 2.

Efficacy

Relapses

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

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performed the ITT analysis as a secondary analysis, to answer a different questions, that were complementary to our core hypothesis, such as; like what happened to all MS patients who were placed on the interventions (the effect of assignment).[45]

Regarding In the per-protocol analysis, during the first year of treatment, the ARR was 0.80, 0.40, 0.78 and 0.83 for the four intervention groups respectively. During the second year, the ARR was 0.90, 0.40, 0.67 and 1.25 for the four intervention groups respectively. Overall, for the two-year primary end point, 8 relapses were recorded for the 10 patients in PLP10 group (0.40 ARR) versus 25 relapses for the 12 patients in on the placebo (1.04 ARR), a 64% adjusted relative rate reduction (RRR) for the PLP10 group (RRR 0.36, 95% confidence interval [(CI]) 0.15 to 0.87, p=0.024) (Tables 3A, 5 and Fig 3A and 3C). After Eexcluding 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, Tables 3B and 5). Pair-wise comparisons for the other two groups against placebo did not yield statistically significant results (Tables 3A, 3B). The proportion of patients with ≤1 relapse for the two years on-study was higher in the PLP10 group than in the placebo group (90% versus 42%, p=0.030, Table 5). Seeking to investigate further investigate the observed difference, we compared the relapse rate during the 24 months before the entry into the study to the 24 months on-treatment for each intervention group. We observed a statistically significant relative reduction in the ARR (70%) only in the PLP10 group (RRR 0.30; 95% CI 0.14 to 0.65, p=0.003, Table 3A); within-group comparisons for the three other groups ARR reduction of the three other groups was were not significant and remained not significant when the natalizumab--treated patients were further excluded from the analysis. The effect of PLP10 through time at different time-windows versus placebo for all-time on-study patients is shown in Figures 3A to 3D. Although theThe ARR analysis, within time-windows, was not

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an assigned endpoint, ~~but~~ it could help ~~in~~ with the process of evaluating parallel information, such as ~~the time needed for a specific treatment intervention activity to be evident, as well as~~ the efficacy profile through time. PLP10 reached its maximum effect within ~~a~~ one year on-treatment (counting from the entry baseline) and remained stable afterwards at an ARR of 0.4~~, displaying a steadily reduced ARR with long~~ with some free-relapse time-windows. ~~These group-B characteristics are considered important parameters of a successful MS treatment where the rule than the exception is the heterogeneity among patients' disease evolution. Specifically,~~ Figure 3D demonstrates the dispersion of relapses throughout the 2-year period of all-time on-study (excluding patients on natalizumab) of PLP10 (n=10) versus placebo (n=10). The p~~P~~lacebo group, in line with the existing knowledge of how relapse history works in relation to future relapses ~~on~~ in MS patients (contagion phenomenon), showed ~~indicates~~ the expected ~~linearly increased~~ trend of ~~the~~ increased relapse incidences.[46] The same phenomenon was true for ~~the~~ groups A and C. Finally, during the 12 month post-study extended period, the ~~(January 1st 2010 to December 31st 2010) all-time~~ on-study patients ~~that~~ who received PLP10~~,~~ showed a persistent benefit in the ARR compared with the~~to~~ placebo (six relapses for the 10 subjects within PLP10 group, 0.6 ARR versus 19 for the 12 subjects within placebo group, 1.58 ARR) indicating a statistically significant 62% adjusted relative rate reduction in the ARR for the PLP10 group (RRR 0.38, 95% CI 0.12 to 0.99, p=0.046).

Regarding the ITT analysis, ~~within PLP10 group, none of the nine drop-out patients changed to natalizumab and two out of nine discontinued because of pregnancy, whereas two out of seven drop-out patients from the placebo group changed to natalizumab (a total of four patients within the placebo arm population were on natalizumab, including the two patients 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] ~~t~~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 8~~96~~% 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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difference was observed for any comparison of the other two groups ~~compared to~~with the placebo group (Fig 4A and Supplementary Information Fig 2).

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

MRI

Over two years, the MRI results support~~the overall conclusion from the study that~~a PLP10-related ~~has a~~ positive effect ~~on disease activity since~~ as only 29% from the PLP10 group, in contrast ~~as opposed~~ to 67% from the placebo group, developed new or enlarging T2 lesions (57% relative risk reduction). After ~~E~~excluding the patients on natalizumab, there ~~is an~~was an increased relative risk reduction (64%) ~~between~~ for PLP10 ~~as opposed~~compared with ~~to~~ the placebo, with 29% of patients on PLP10 and 80% on placebo ~~with~~ develop~~ment~~ing ~~of~~ new or enlarging T2 lesions (Table 5).

Safety

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

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~~reason~~aetiology for the drop-outs was the palatability and smell of the formula preparations in addition to pregnancy. Nausea was reported by two patients. No abnormal values were observed on any of the biochemical and haematological blood tests. No allergic reactions were reported.

Discussion

In this proof-of-concept randomised, double-blind clinical trial assessing the safety and ~~clinical trial assessing the safety and~~ efficacy of three variations of a novel ~~cocktail~~ nutritional~~intervention~~ formula~~s~~ in RRMS, we observed a significant ~~benefit~~ association for ~~the novel the~~a formula containing a balanced mixture of specific omega-3 and omega-6 PUFAs, MUFAs, SFAs, vitamin A, vitamin E and γ-tocopherol ~~.....~~ (PLP10) ~~intervention~~ compared ~~to~~ with the placebo for both the ARR and the progression to disability in the per-protocol analysis. Our results included analyses pertaining to a total of 42 months of study-collected data, including the 12-month intervention-free treatment extension period. ~~Our results include analyses pertaining to a total of 42 months study-collected data, including the 12-month, free of intervention treatment, extension period. We focused on the per-protocol data analysis since it is the appropriate method to best provide the answer to the proof-of-concept trial addressed question.~~ We also observed, a~~The~~ high drop-out rate that was mostly the result of formula~~s~~ palatability, a common phenomenon in trials using oily interventions ~~where a lot of patients tend to drop-out soon after first dosage~~. ~~We thus present our main per-protocol analysis, as well as a subgroup analysis excluding patients on natalizumab.~~ Interestingly, a ~~We have found a~~ statistically significant reduction in the ARR and ~~the~~ disability progression was also observed when ~~comparing not only patients on PLP10 versus placebo but also~~ comparing the ARR of the PLP10 patients in the 24-month period prior to the study ~~to~~ with the ARR of the 24 months on-study; the observed differences became

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

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

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

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known that the majority of the patients suffering from MS do use dietary supplements for a variable length of time.[52] Dietary antioxidants and fatty acids may influence the disease process in MS by reducing immune-mediated inflammation, oxidative stress and excitotoxic damage.[12] Published data have revealed that healthy dietary molecules have a pleiotropic role and are able to change cell metabolism and down-regulate inflammation by interacting with enzymes, nuclear receptors and transcriptional factors.[51] Current available treatments are the products of reductionism, partially effective and associated with severe side effects. Interferons and glatiramer acetate, the most widely used first-line MS drugs available today, are associated with the least severe side effects among the MS therapies, but they are reported to reduce the ARR only by about one third and with no significant effect on the progression of disability.[53] Natalizumab reduces the ARR by 68% and decreases the possibility of disability progression by 43%, with 57% of patients free of new or enlarging T2 lesions on MRI scans, compared with 15% on placebo.[54] Fingolimod is associated with a 54% ARR reduction (without a significant benefit on the progression of disability). Both natalizumab and fingolimod are second-line drugs associated with severe side-effects.[55]

~~efficacy of a~~ ~~After a thorough search in the literature we are convinced that no existing MS treatment module has ever been designed according to as a result of the SM systems medicine concept approach, or with a potential to effectively stimulate intrinsic remyelinating and neuroprotecting mechanisms or exert such an action.~~ ~~Now w~~ Mehta in a review paper, in 2009, reported different clinical studies on interventions formulated based on the individual aforementioned molecular ingredients or based on a specific ratio of the aforementioned molecular ingredients for MS treatment; although no one was reported using the antioxidant vitamin γ-tocopherol.[56] In our study, the choice of ingredient proportion and dosing scheme was based upon evidence derived form *in vivo* and *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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populations that consume fish-based diets, the ratio is about 1:1–2.[52, 53] The intervention daily dose was designed aiming and believed to be high enough to restore/amplify body efficient antioxidant activity and ensure cellular membranes lipid profile normalization (PUFA content) and simultaneously potentiate involvement of the ingredients in the anti-inflammatory and recovery mechanisms. Diet fatty acid molecules need about a six months period to exert their beneficial effect and this essential parameter was for the first time under consideration in our study design (normalization period).[42] This chronotherapy parameter might be of major importance and is in line with the systems medicine treatment philosophy. We believe that the persistent effect within the post-study period is in agreement with the reported very long washout phase for omega-3 fatty acids, especially DHA, to return to the pre-treatment values.[46] Considering that omega-3 supplementation can release and replace excess AA within the cellular membranes, we can speculate that an increased inflammatory activity can possibly result during the first six months of supplementation.

In addition to the EPA, DHA, LA, and GLA, PLP10 contained limited quantities of other structural/active PUFAs, specific MUFAs (mostly oleic acid) and SFAs (palmitic and stearic acid), specifically to provide a direct source for neuronal cell membranes rehabilitation and for (re)myelination and neuroprotection because these compounds are all major components, precursors and building blocks of any new physiological myelin and cellular membranes in general. Assembly of the correct molecules into the myelin membrane may be especially critical during active synthesis. If these critical constituents aren not directly or indirectly available, amyelination, dysmyelination or demyelination may ensue.[56] The maintenance of myelin requires continued turnover of its components throughout life.[54,55]

Different factors and molecular entities appear to be part of the possible aetiology for MS, with specific PUFA and antioxidants found to be key substances related to all known pathogenic and recovery mechanisms. In our study, we further proposed that a holistic systems medicine model approach can be applied by synchronized action. First, there is an obvious convenience in administering one formula containing different specific active ingredients. The currently available evidence supports that nutritional interventions would confer a small to medium treatment effect with an accompanying appropriate safety profile.[12 52 56] Combining these specific active ingredients together with γ-tocopherol and other specific active molecules into one stable formulation is expected to enhance adherence while still offering an appropriate safety profile. A similar approach could not be adopted for pharmaceutical interventions with common and severe adverse events, such as those indicated today for patients with MS. Given the advantages of the simultaneous use and that all the included ingredients have proven individually a valid biological plausibility and have been tested in various settings and under various dose schemes, we also assessed the hypothesis that a novel mixture of these ingredients would have a postulated efficacy attained synergistically through different mechanisms of action.[52 56] Interestingly, the observed magnitude of the treatment effect cannot be explained by adding up the postulated efficacy estimates of the individual ingredients. Findings from *in vitro* and *in vivo* studies support this notion of proposed synergy although this hypothesis can only be taken forward when the observed treatment effect is validated in various settings and in a larger number of patients.

We acknowledge that our study has two considerable limitations: the small sample size and the high drop-out rate. Regarding the sample size, one should bear in mind that this study is a small, phase-2 clinical trial assessing a novel intervention and thus has comparable size in the appropriate literature. Questions taken forward from this trial can be assessed in a larger

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randomised trial in which appropriate power calculations would be possible, taking into consideration the findings of the present study. The adherence of the subjects is another limitation of our study, but the total duration of the study that covers a total of 42 months follow-up adds power to the results.[48] We acknowledge that we had to deliver the intervention in the way most frequently associated with low compliance, i.e., an oral, liquid formula, thus triggering maximum intolerance due to taste. Nevertheless, the observed suboptimal compliance is in accordance with the published literature in which clinical trials assessing liquid fatty acid interventions show a weaker adherence compared with clinical trials of pharmaceutical interventions. Indeed, in our study, we consistently recorded the reasons for withdrawal: most of the participants did not discontinue due to safety issues, but rather due to palatability issues. Controlling non-compliance due to palatability issues is by far easier to address compared with non-compliance related to adverse events and can be resolved when optimisation of the formulation is achieved in future trials. At this stage of the development of the intervention, we would by far exceed the cost-effectiveness threshold if we were to invest in improving these features of the intervention. Moreover, we should also note that MS patients are subject to far more frequent and more serious adverse events related to the current standard treatments.

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

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

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

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~~normalization of all cellular membranes lipid content. The intention is to normalize the specific nutritional requirements of the MS patients.~~

~~Interestingly, in this small phase II trial, we observed a far larger treatment effect than expected. One explanation could be maximum synergistic effect observed when stimultaneously administering the assessed ingredinets. Findinds from in-vitro and in-vivo studies could support this notion. ... Nevertheless, this hypothesis can only be taken forward when Different factors and molecular entities appear to be part of the possible aetiology for MS with specific PUFA and antioxidants found to be key substances related to all known pathogenic and recovery mechanisms. But, it is well established that MS patients are characterized with significant deficiencies in antioxidant efficiency as well as in PUFA levels in blood and cellular membranes.[11, 49-51]~~

~~According to one hypothesis, the change in the ratio of omega-3 to omega-6, due to the increasing consumption of omega-6 PUFA and resulting in, meaning high accumulation of AA, in the Western diet, may be one of the major factors responsible for the increasing incidence of inflammatory diseases relative to populations. In the Western diet, the ratio of omega-3 to omega-6 is about 1:20–30; in populations that consume fish-based diets, the ratio is about 1:1–2.[52, 53] The intervention daily dose was designed aiming and believed to be high enough to restore/amplify body efficient antioxidant activity and ensure cellular membranes lipid profile normalization (PUFA content) and simultaneously potentiate involvement of the ingredients in the anti-inflammatory and recovery mechanisms. Diet fatty acid molecules need about a six months period to exert their beneficial effect and this essential parameter was for the first time under consideration in our study design (normalization period).[42] This chronotherapy parameter it is of major importance in line with the SM treatment philosophy~~

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We believe that the persistent effect within the post-study period is in agreement with the reported very long washout phase for omega-3 fatty acids, especially DHA, to return to the pre-treatment values.[46] and if it is not included in the trial design the possibility of misleading result evaluation greatly increases. In fact, considering that omega-3 supplementation can release and replace excess AA within the cellular membranes, we can speculate that an increased inflammatory activity can possibly result during the first six months of supplementation (during normalization period).

The maintenance of myelin requires continued turnover of its components throughout life.[54,55] In addition to the EPA, DHA, LA, and GLA, PLP10 was containing limited quantities of other structural PUFA, specific MUFA (mostly oleic acid) and SFA (palmitic and stearic acid), specifically aiming to provide a direct source for neuronal cell membranes rehabilitation and for (re)myelination and neuroprotection since they are all major components, precursors and building blocks of any new physiological myelin and cellular membranes in general. Assembly of the correct molecules into myelin membrane may be especially critical during active synthesis. Possibly, if critical constituents aren't available or are metabolically blocked, amyelination, dysmyelination or demyelination may ensue.[56]

We acknowledge that our study has two considerable limitations pertaining to the small sample size and the high drop-out rate. Regarding the sample size, one has to bear in mind that this is a small, phase 2 clinical trial assessing a novel intervention and has thus comparable size in the relative literature. Questions taken forward from this trial can be assessed in a larger randomized trial where appropriate power calculations would be possible taking into consideration the findings of the present study. The adherence of the subjects is another limitation of our study since almost half of the participants withdrew. We

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acknowledge that we had to deliver the intervention in the way most frequently associated with low compliance, i.e. oral, liquid formula, thus triggering maximum intolerance due to taste. Nevertheless, the observed suboptimal compliance is in accordance with the published literature where clinical trials assessing liquid fatty acid interventions show a weaker adherence compared to clinical trials of pharmaceutical interventions. Indeed, in our study, we consistently recorded the reasons of withdrawal and most of the participants did not discontinue due to safety issues, but mostly in relation to palatability issues. Controlling non-compliance due to palatability issues is by far easier to deal with compared to non-compliance related to adverse events and can be resolved when optimization of the formulation is achieved in future trials. At this stage of the development of the intervention, we would by far exceed the cost-effectiveness threshold if we were to invest in improving these features of the intervention. Moreover, we should also note that MS patients are subject to far more frequent and more serious adverse events related to the current standard treatment.

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

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

The present preliminary, small size, randomized, controlled phase II clinical trial provides evidence for a novel ~~nutritional~~nutraceutical formula based on dietary, metabolic, immunological, and neurobiological pathways possibly involved with disease progression in MS. This novel intervention showed signs of efficacy in the observed annualised relapse rate~~s~~ and disability progression ~~to disability.~~ ~~.~~We took the appropreate~~all~~ methodological ~~precautions~~measures ~~in order to~~to control for potential sources of bias and ~~be~~to enable ~~able to reach~~ a valid interpretation to be reached. We acknowledge that the presence of bias can only be minimized, ~~yet~~ not excluded, in any clinical research setting and also that random error is always a possible scenario in small trials. Thus, we present the observed results as an additional piece of randomized evidence and anticipate the replication of our study findings in a larger randomised controlled clinical trial.

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

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another issue but the duration of the study (42 months) is adding power to the results;[44] having the research questions been consciously and carefully approached and answered. Furthermore, the statistical methodologies used along with the appropriate adjustments, broadly accepted for MS clinical trials, power strengthen the findings, results, and significance. The baseline characteristics of the treatment arms could possibly be considered indicative of four very active groups of patients but that was the result of the limited number of RRMS population eligible for the study within Cyprus. On the other hand the balanced baseline characteristics without statistical differences, the statistical adjustments (for all important baseline parameters i.e. relapses, EDSS, age and DMT) and the randomization within four different groups are the safety valves against data misinterpretation. Yet, in small randomized control trials with a high drop-out rate, the per-protocol analysis could be affected by the characteristics of the patients dropping out. In order to safeguard our findings in the best possible way under the circumstances, we proceeded to adjusting for confounders. Moreover, we cannot discard our finding as a false positive, given that this is a randomized, double-blind, placebo-controlled clinical trial and, despite its small sample size, represents a piece of evidence that only a larger randomized controlled trial can replicate or refute. It is possible to question why DMTs efficacy cannot be emerged out of the data analysis, of the four treatment arms, and in accordance to their published values. We believe that the limited efficacy of the DMTs, the sample size and the statistical adjustments were strong limiting determining factors for such an indication to be countable. An additional argument is that the efficacy reported for the analysis of pre-treatment (24 months before entry baseline) versus on-trial ARR could be considered as potentially biased due to differences of how relapses were defined during the course of a study compared to pre-treatment period; or due to regression to the mean or placebo effect. This analysis was performed as an additional exploratory analysis that we were able to do due to the availability of data. The relapses of

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

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

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

~~It is commonly accepted that nutrition is one of the possible environmental factors involved in the pathogenesis of MS, but its role as complementary MS treatment is unclear and largely disregarded.[57] It is well known that the majority of the patients suffering from MS they do use dietary supplements for a variable length of time and they prefer supplement type of "help" over conventional drugs.[58] Dietary antioxidants and fatty acids may influence the disease process in MS by reducing immune-mediated inflammation, oxidative stress and excitotoxic damage.[11] Present data reveal that healthy dietary molecules have a pleiotropic role and are able to change cell metabolism from anabolism to catabolism and down-regulate inflammation by interacting with enzymes, nuclear receptors and transcriptional factors.[57] The present preliminary small size randomized controlled phase II clinical trial, for the first time provides link evidence between dietary, metabolic, immunological, and neurobiological aspects of MS after three quarters of a century of unsuccessful scientific efforts. This link evidence might probably be the beginning of opening new horizons and new avenues in the approach of MS prevention and treatment, and possibly of 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) 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 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 1year**	**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 by1 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 by1 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
† 1out 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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for the protocol and study design. M.C.P was the evaluator of patients' clinical progress signs and the treatment physician. I.S.P and G.N.L were involved in the non-pharmacologic treatment-related care. I.S.P performed the literature search and both I.S.P and G.N.L contributed on the intervention formulation and composition rational. I.S.P supervised the composition procedure of the interventions and the fatty acid profile analysis of the red blood cell membranes. E.E.N and I.S.P performed the statistical analyses. E.E.N was an independent scientist. All authors vouch for the accuracy and completeness of the data and the statistical analyses.

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Ethical approval: Cyprus National bioethics committee (reference EEBK/EII/2005/10).

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All authors have completed the Unified Competing Interest form at www.icmje.org/coi_disclosure.pdf (available on request from the corresponding author) and declare that (1) M.C.P, G.N.L, I.S.P have support from Cyprus Ministry of Commerce, Industry and Tourism, Program for the Creation of New High Technology and Innovation Enterprises through the Business Incubator for the submitted work; (2) E.E.N has no relationships with Cyprus Ministry of Commerce, Industry and Tourism, or PLUPA Medical Ltd. that might have an interest in the submitted work in the previous 3 years; (3) their spouses, partners, or children have no financial relationships that may be relevant to the submitted work; and (4) E.E.N has a non-financial interests that may be relevant to the submitted work.

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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 legends

Figure 1. Study Flowchart

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

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

Abbreviations: PL, phospholipid; IFN-γ, interferon γ; IL-2, interleukin 2; NFκB, nuclear factor kappa B; PGE2, prostaglandin E2; PPARγ, peroxisome proliferator-activated receptor γ; PUFAs, polyunsaturated fatty acids; TGFβ, 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.

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

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

Panel D demonstrates the dispersion of relapses throughout the 2-year period of all-time on-study (excluding patients on natalizumab) of PLP10 (n=10) *vs.* placebo (n=10). Placebo shows an irregular dispersion of relapses in relation to PLP10 group with linear increasing trend wile PLP10 shows a stabilized linear trend. By using the per-protocol model where patients on natalizumab were excluded, we could compare the number of relapses on a same number of patients.

** Including the patients on natalizumab.

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

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

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Figure 5. Mean change in expanded disability status scale score as a function of visit number. Values are expressed as mean ± standard error of the mean (s.e.m.)

¶ Including patients on natalizumab

¶¶ Excluding patients on natalizumab

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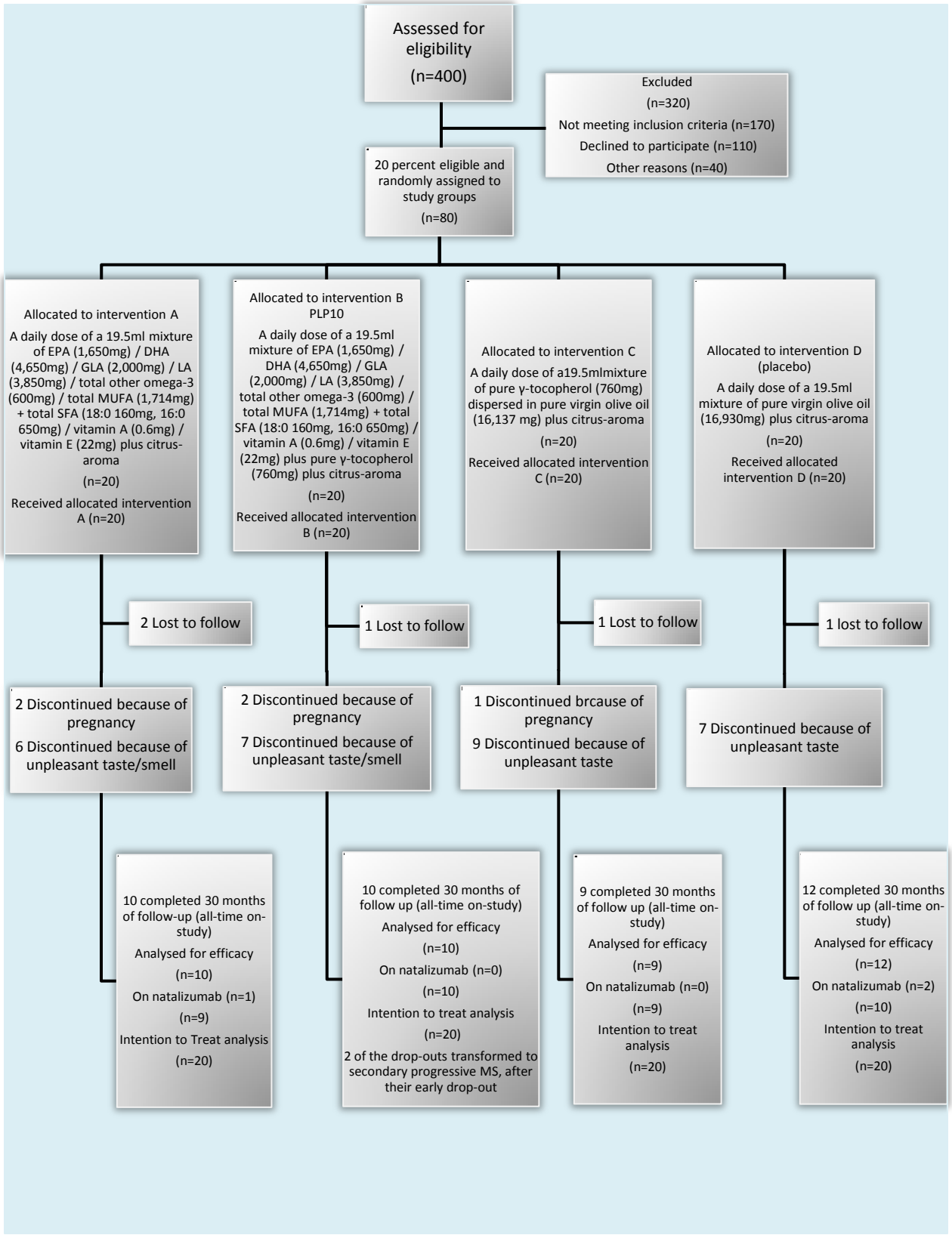
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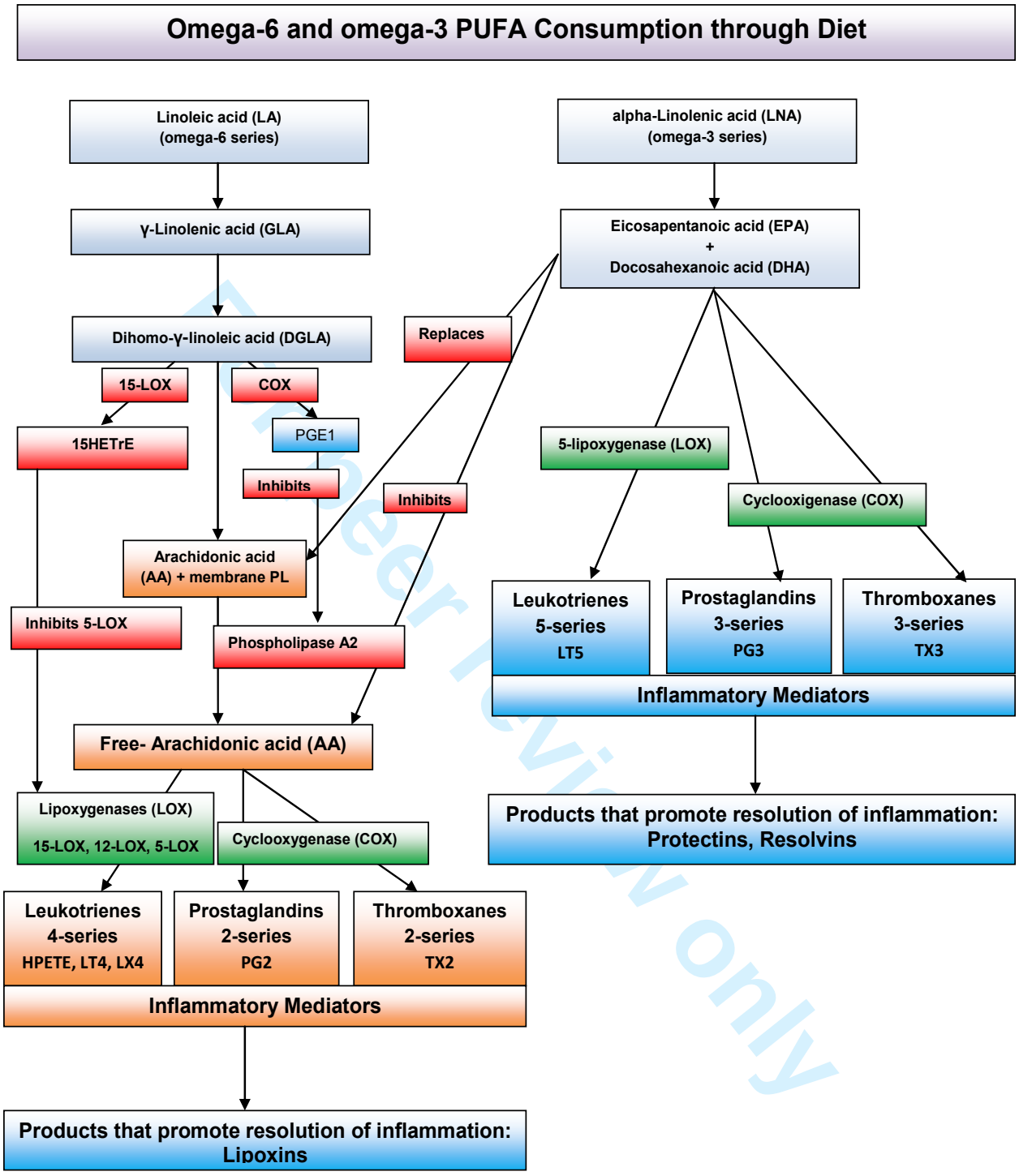
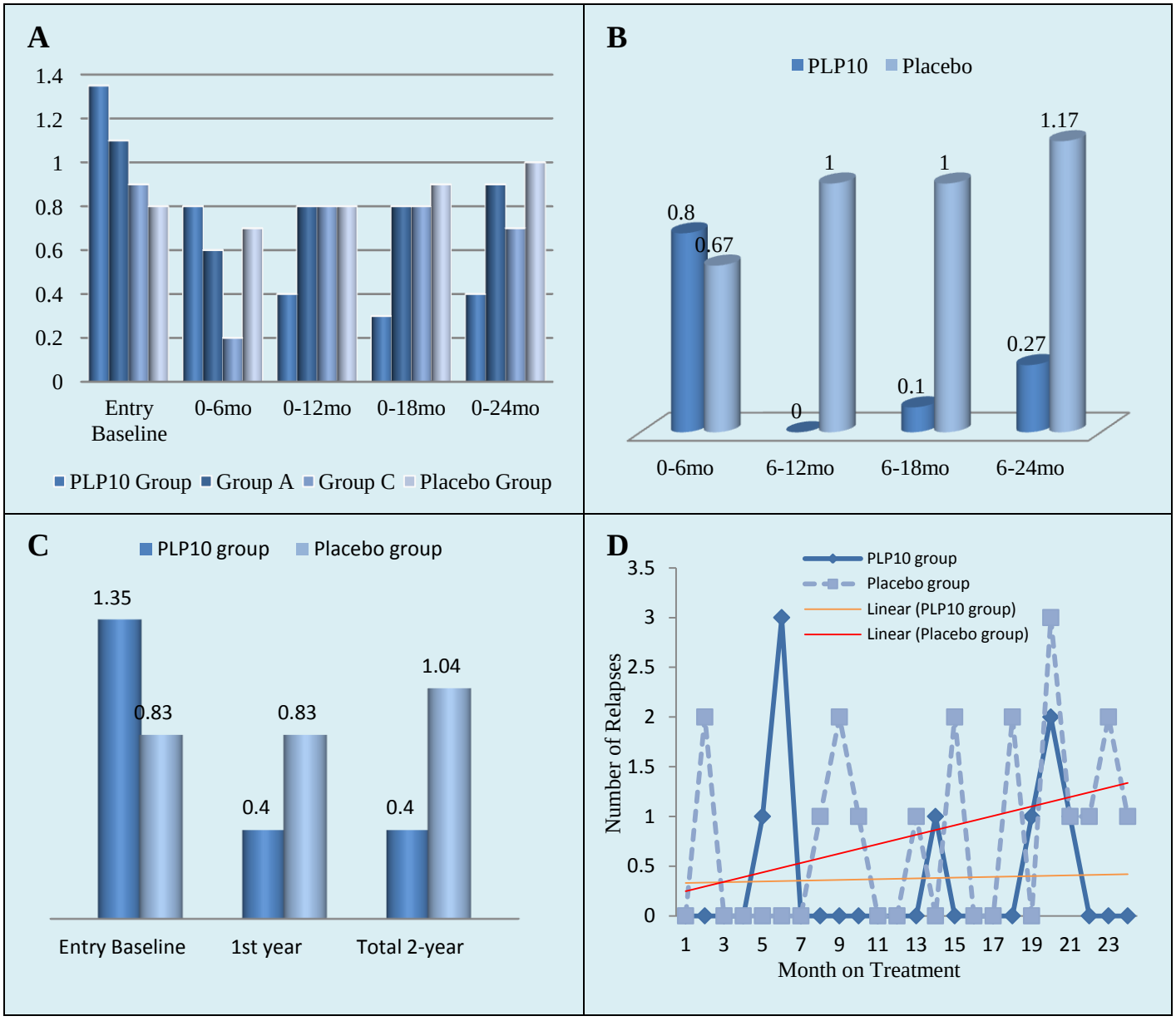
(n=400)
Excluded
(n=320)
Not meeting inclusion criteria (n=170)
Declined to participate (n=110)
Other reasons (n=40)
20 percent eligible and randomly assigned to study groups
(n=80)
Allocated to intervention A
A daily dose of a 19.5ml mixture of EPA (1,650mg) / DHA (4,650mg) / GLA (2,000mg) / LA (3,850mg) / 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
(n=20)
Received allocated intervention A (n=20)
Allocated to intervention B PLP10
A daily dose of a 19.5ml mixture of 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 pure γ-tocopherol (760mg) plus citrus-aroma
(n=20)
Received allocated intervention B (n=20)
Allocated to intervention C
A daily dose of a19.5mlmixture of pure γ-tocopherol (760mg) dispersed in pure virgin olive oil (16,137 mg) plus citrus-aroma
(n=20)
Received allocated intervention C (n=20)
Allocated to intervention D (placebo)
A daily dose of a 19.5ml mixture of pure virgin olive oil (16,930mg) plus citrus-aroma
(n=20)
Received allocated intervention D (n=20)
2 Lost to follow
1 Lost to follow
1 Lost to follow
1 lost to follow
2 Discontinued because of pregnancy
6 Discontinued because of unpleasant taste/smell
2 Discontinued because of pregnancy
7 Discontinued because of unpleasant taste/smell
1 Discontinued brcause of pregnancy
9 Discontinued because of unpleasant taste
7 Discontinued because of unpleasant taste
10 completed 30 months of follow-up (all-time on-study)
Analysed for efficacy
(n=10)
On natalizumab (n=1)
(n=9)
Intention to Treat analysis
(n=20)
10 completed 30 months of follow up (all-time on-study)
Analysed for efficacy
(n=10)
On natalizumab (n=0)
(n=10)
Intention to treat analysis
(n=20)
2 of the drop-outs transformed to secondary progressive MS, after their early drop-out
9 completed 30 months of follow up (all-time on-study)
Analysed for efficacy
(n=9)
On natalizumab (n=0)
(n=9)
Intention to treat analysis
(n=20)
12 completed 30 months of follow up (all-time on-study)
Analysed for efficacy
(n=12)
On natalizumab (n=2)
(n=10)
Intention to treat analysis
(n=20)



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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0-24mo
PLP10 Group
Group A
Group C
Placebo Group
B
PLP10
Placebo
0.8
0.67
1
0
1
0.1
1.17
0.27
0-6mo
6-12mo
6-18mo
6-24mo
C
PLP10 group
Placebo group
1.35
0.83
0.4
0.83
0.4
1.04
Entry Baseline
1st year
Total 2-year
D
PLP10 group
Placebo group
Linear (PLP10 group)
Linear (Placebo group)
Number of Relapses
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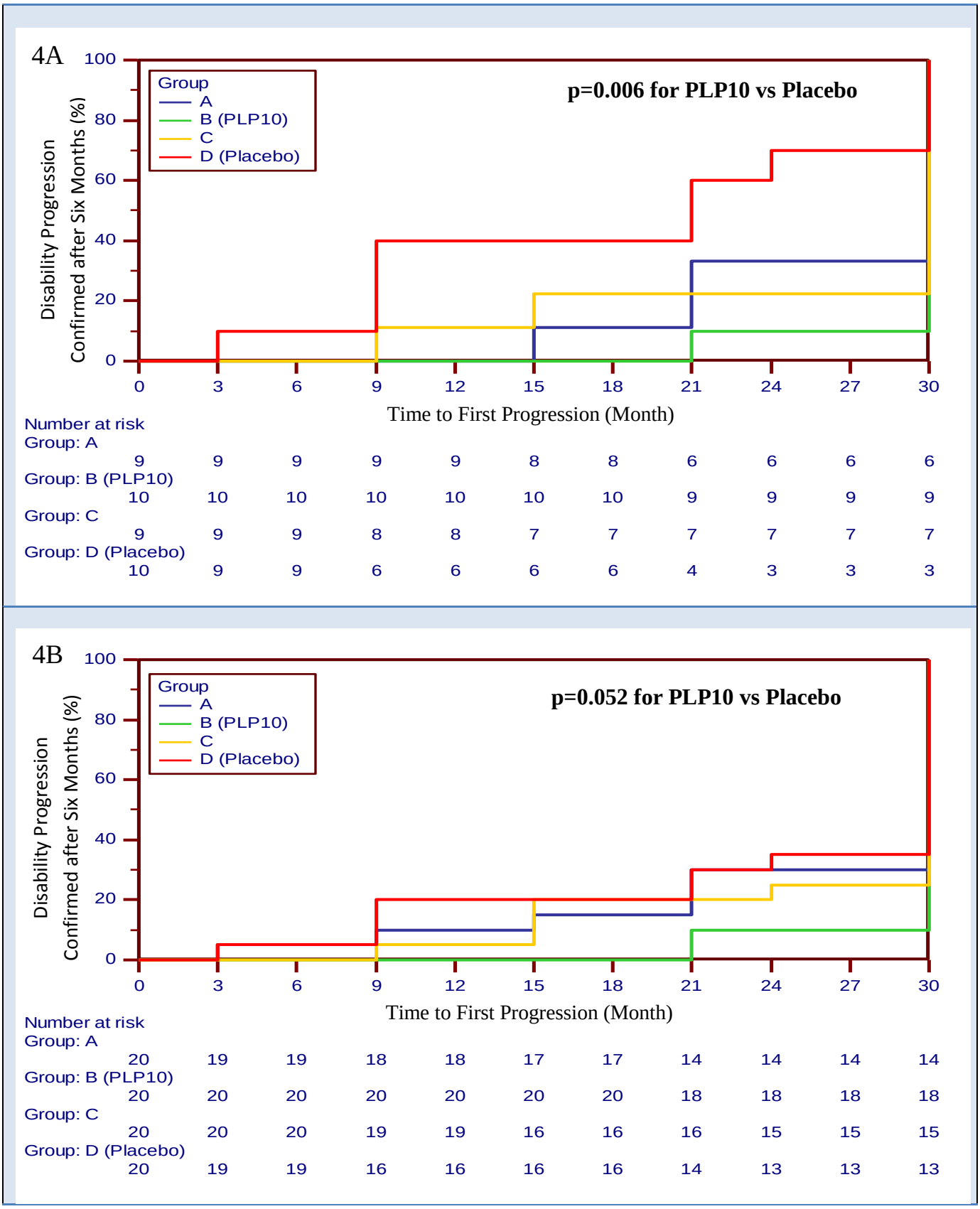
Month on Treatment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Group
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B (PLP10)
C
D (Placebo)
p=0.006 for PLP10 vs Placebo
Disability Progression
Confirmed after Six Months (%)
100
80
60
40
20
0
0 3 6 9 12 15 18 21 24 27 30
Time to First Progression (Month)
Number at risk
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
4B
Group
A
B (PLP10)
C
D (Placebo)
p=0.052 for PLP10 vs Placebo
Disability Progression
Confirmed after Six Months (%)
100
80
60
40
20
0
0 3 6 9 12 15 18 21 24 27 30
Time to First Progression (Month)
Number at risk
Group: A
20 19 19 18 18 17 17 14 14 14 14
Group: B (PLP10)
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Group: C
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Group: D (Placebo)
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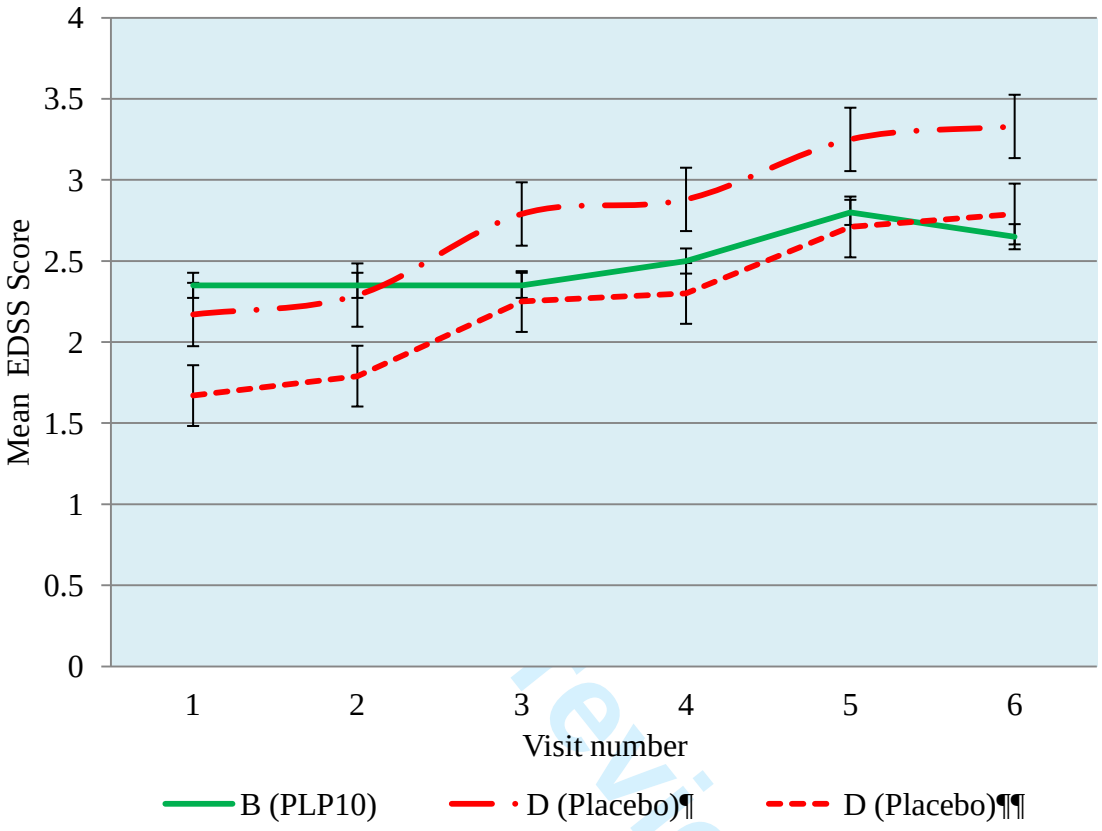

Supplementary Information

Table of Content

Supplementary Information Methods 1

Intervention and study rational. We are using high dosage of 1:1 omega-3:omega-6 polyunsaturated (PUFA) aiming to overpass and normalize global diet regional traditions and habits in relation to abnormal PUFA/saturated fatty acids (SFA)/monounsaturated fatty acids (MUFA) daily ratio consumption irrespective of the quantities consumed; and enough to equilibrate patients' diet in line with the recommended more physiologic omega-3:omega-6 ratio of about 1:1-4 wt/wt as reported by Simopoulos, 2002. In addition to correct existing deficiencies, cell membrane abnormalities, specifically of the immunopathological system and blood mononuclear peripheral cells, and high enough for availability and immediate ongoing modulation of the involved pathogenic mechanisms and network of events in MS. The high dosage is also required to overpass the quantity limitations, previously discussed, of diet-consumed PUFAs for cellular incorporation, especially in the central nervous system (CNS) of adults. Additionally, fatty acids (FAs) must first cross the intestinal epithelium before reaching the different tissues, where digestion and absorption constitute further problems in their availability (Carlier, H, 1991). Omega-3 PUFA are used in re-esterified form to eliminate unwanted disturbances, at the sides of action, by other fatty acids and molecules present in crude fish oils but also to increase the bioavailability of the FA since triglycerides have been shown to be associated with much higher bioavailability (Dyerberg et al, 2010). Linoleic (LA) and gamma linolenic acid (GLA) are essential structure molecules and important for any physiological (re)generation of cell membrane. GLA quantity is doubled to LA to ensure high direct production of dihomo-gamma-linolenic acid (DGLA), from GLA when LA cannot be metabolized, due to desaturase deficiency or malfunction. Such a reduced capacity to convert LA to GLA has been associated with aging, diabetes, alcoholism, atopic dermatitis, premenstrual syndrome, rheumatoid arthritis, cancer and cardiovascular diseases (Bolton-Smith et al, 1997; Horrobin, 1990; Leventhal et al, 1993). This is going to result in the increase of DGLA relative to arachidonic acid (AA) with DGLA promoting production of prostaglandin (PG)E1 but also inhibition of phospholipase (PL)A2: two major reasons and rational for their use. If other metabolic problems are involved within the omega-6 series and the normal metabolites are not produced then the eicosapentaenoic acid EPA available in PLP10 will substitute the function of DGLA, as a competitive inhibitor of AA for PLA2. In both cases the pro-inflamatory leucotrienes, prostaglandines of the 2-series (PG2) and thromboxanes of the 2 series including the platelet-activator factor (PAF) will be attenuated. The synthesis of AA from DGLA by Δ5 desaturase promoted by LA/GLA supplementation is very limited in humans as a result of limited activity of the enzyme (Yang-Yi & Robert, 1998). AA in the body is mostly available through diet. EPA and docosahexaenoic acid (DHA) are both physiologically important and crucial structured molecules able to substitute excess AA and SFA within the cell membranes. EPA will contribute to the inhibition (competitive to AA) of PLA2, joining the co-supplied omega-6 PUFA but will also participate in the production of anti-inflammatory leukotrienes, prostaglandins of the 3-series (PG3) and thromboxane (TX3) along with DHA, both found in the PLP10 intervention. Moreover EPA will replace AA of the membrane phospholipids and both omega-3 PUFA will contribute replacing abnormal quantities of SFA and excess AA. DHA is used in 3:1 ratio to EPA to cover any possible inabilities of EPA to be metabolized, high enough to strongly promote high production of the aforementioned anti-inflammatory

eicosanoids and cytokines and to be incorporated into CNS cell membranes where DHA should be the major PUFA present, replacing other FA, probably saturated and excess of AA. EPA, DHA, LA and GLA along with the rest of the other ingredients used ("other" omega-3 PUFA, SFA and MUFA that are usually found in the cell membranes of healthy people in limited quantities) in the intervention regimen are for their availability as minor structural constituents of physiological cellular membranes integrity, fluidity and overall function as building blocks for myelin repair and/or myelination. Furthermore the PUFA used within the cocktail intervention aimed to manipulate all other pathophysiological pathways that are reported to be able to: as previously discussed including gene transcription for neuroprotection and remyelination. Furthermore, PUFA are used to ensure the integrity of blood brain barrier (BBB) and to modulate the gelatinases responsible for the T cell migration within the CNS. Three different antioxidant vitamins (vitamin E, mostly as alpha-tocopherol, gamma (γ)-tocopherol (vitamin E isoform) and vitamin A) are used in the regimen preparation to support the cellular antioxidant defenses but also to protect peroxidation of the supplied increased amounts of PUFA. Alpha-tocopherol low-molecular-weight antioxidants will contribute to radical scavenging, interfering with gene transcription, protein expression, enzyme activity and metal chelation (van Meeteren et al, 2005). Vitamin E (alpha tocopherol) and vitamin A are used as antioxidants for the protection of the excess supplemented PUFA, with alpha-tocopherol been demonstrated to protect against peroxynitrite-induced oxidative damage, as well as able to efficiently detoxify hydroxyl, perhydroxyl and superoxide free radicals (the elevated reactive oxygen species (ROS)); each one with different mechanism of action, increasing the effect capability (Vatassery et al, 1998b; van Meeteren, 2005). Gamma-tocopherol is used in high dosage since its half life is very short compared to alpha-tocopherol and has been demonstrated to specifically protect against nitro-radicals. Tocopherols can also exert non-antioxidant properties, including modulation of cell signaling and immune function, regulation of transcription, and induction of apoptosis as previously discussed (van Meeteren et al, 2005).

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

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

adjunct to existing pharmaceuticals produced by reductionism, can be proven therapeutically superior to any available treatment for MS.

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Carlier H, Bernard A, Caselli C (1991) Digestion and absorption of polyunsaturated fatty acids. *Reproduction, nutrition, development* **31:** 475-500

Dyerberg J, Madsen P, Møller JM, Aardestrup I, Schmidt EB (2010) Bioavailability of marine n-3 fatty acid formulations. *Prostaglandins, leukotrienes, and essential fatty acids* **83:** 137-141

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Leventhal LJ, Boyce EG, Zurier RB (1993) Treatment of rheumatoid arthritis with gammalinolenic acid. *Annals of Internal Medicine* **119:** 867-873

Simopoulos AP (2002) The importance of the ratio of omega-6/omega-3 essential fatty acids. *Biomedecine & Pharmacotherapy* **56:** 365-379

van Meeteren ME, Teunissen CE, Dijkstra CD, van Tol EAF (2005) Antioxidants and polyunsaturated fatty acids in multiple sclerosis. *Eur J Clin Nutr* **59:** 1347-1361

Yang-Yi F, Robert SC (1998) Importance of Dietary g-Linolenic acid in human health and nutrition. *The Journal of Nutrition* **128:** 1411-1414

Vatassery GT, Smith WE, Quach HT (1998b) Alpha-tocopherol in rat brain subcellular fractions is oxidized rapidly during incubations with low concentrations of peroxynitrite. *Journal of Nutrition*128:152–157.

Supplementary Information Methods 2

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

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

The daily intervention formula agent dosages were:

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

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

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

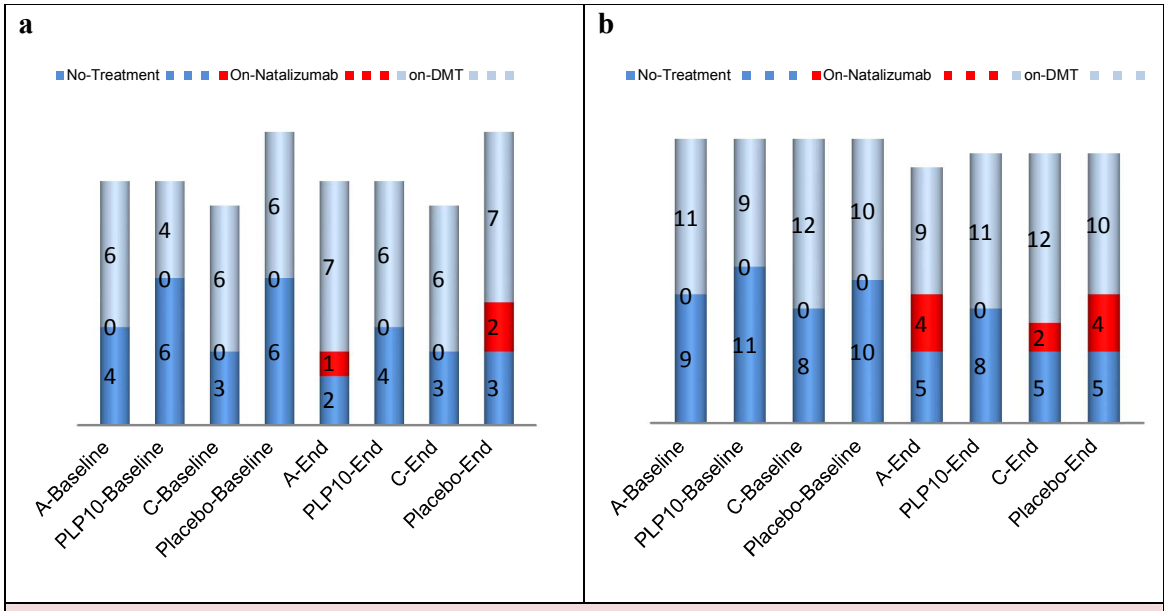
Intervention formula D daily dosage**:** pure virgin olive oil (16930mg).

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

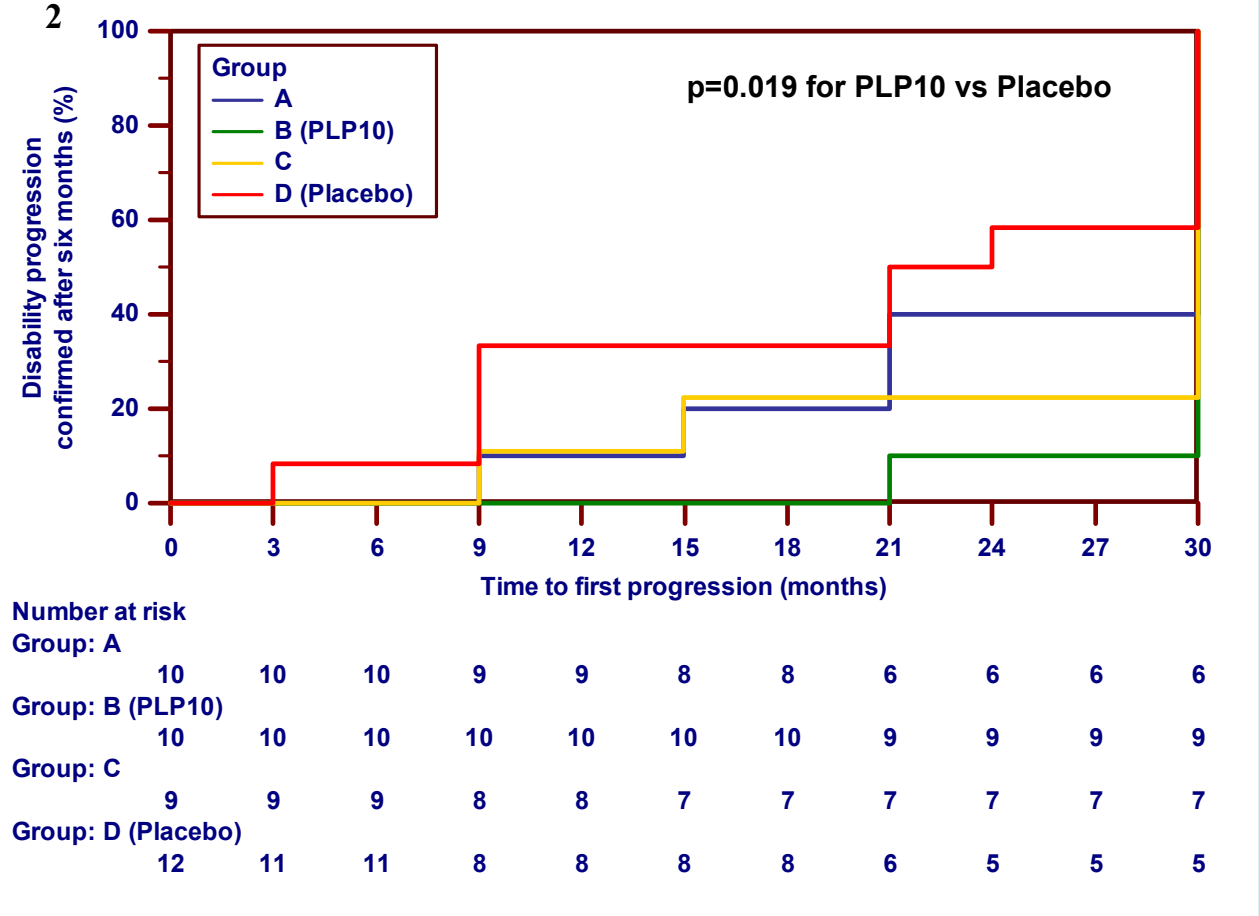
The specific omega-3 related fraction, according to specifications required for the interventions was prepared and purchased from EPAX AS, Aalesund, Norway; as re-esterified glycerides from fish body oils as a source. The specific omega-6 PUFA, MUFA and SFA related fraction, according to required specifications,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

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

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



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.



Supplementary Information Figure 2 | Kaplan–Meier estimates for the time to disability progression. Kaplan–Meier plot of the time to sustained progression of disability among all-time on-study patients, including patients on natalizumab, receiving intervention A, PLP10 and C *vs.* placebo. Intervention PLP10 reduced the risk of sustained progression of disability by 83% over two years (p=0.019). The cumulative probability of progression was 10% in the intervention B group and 58% in the placebo group. Intervention formula A reduced the risk of sustained progression of disability by 32% (p=0.301) and intervention formula C by 62% (p=0.109).

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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.