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BENEFICIAL EFFECTS OF A NANO FORMULATION OF POMEGRANATE SEED OIL, GRANAGARD, ON THE COGNITIVE FUNCTION OF MULTIPLE SCLEROSIS PATIENTS

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Highlights

- No adverse events were recorded during a 3-month treatment of patients with MS with GranaGard, a brain targeting nano-formulation of PSO.
- GranaGard administration seems to induce short-term beneficial effects and to improve/stabilize cognitive disability in MS patients.
- Putative mechanisms of action of Granagard are mostly related to its strong anti-oxidative effects.

ABSTRACT

Background: Multiple sclerosis (MS) is a chronic, progressive disease of the central nervous system (CNS) that is characterized by inflammatory damage to the myelin sheath. Though often neglected, cognitive impairment is a common feature of MS that affects 43-70% of patients. None of the novel MS treatment seems to substantially affect or restore cognitive disability in MS. GranaGard (Granalix Bio Technologies LTD) is a food supplement shown in animal studies to prevent neuronal death in several models of neurological diseases. Capsules of GranaGard comprise a self-emulsion nano formulation of pomegranate seed oil (PSO). This oil contains 80-90% of Punicic Acid (PA), one of the strongest natural antioxidants. In animal experiments, administration of GranaGard results in conjugation with linoleic acid (CLA), the main metabolite of PA, which is a well-known neuroprotective agent.

Aims: To investigate whether GranaGard administration has an effect on the cognitive state of MS patients.

Methods: This is a single center, randomized double blind study that started in May 2018. The study included 30 MS patients; half of them (Group-A) were given GranaGard for the first three months and then placebo pills containing soybean oil for additional three months. Patients in Group-B received placebo for the first three months, and GranaGard for the following three months. GranaGard was administrated in addition to their immunomodulatory MS-treatments. Subsequently, all patients received GranaGard for additional six months. Patients were required to visit the doctor at baseline (inclusion, visit 1) and at 3 months after treatment-initiation at each cycle of the experiment (visits 2 and 3). During the follow up visits, clinical and cognitive examinations were performed, including Expanded Disability Status Scale (EDSS), Multiple Sclerosis Functional Composite (MSFC: 25ft walking test, 9 PEG hole test & PASAT). Cognitive examinations included The Brief International Cognitive Assessment for Multiple Sclerosis (BICAMS) battery: 1) Symbol Digit Modalities Test (SDMT); 2) California Verbal Learning Test – Second Edition (CVLT-II); 3) and Brief Visuospatial Memory Test – Revised (BVMT-R). Cognitive outcomes were normalized to the healthy population and expressed as z-scores, depended on age, gender and education. Short quality of life and fatigue questionnaires (SF-12, MFIS-5) were also provided by the participants.

Results: No serious side effects, related to the product, were observed during the study period. All patients receiving GranaGard reported a ''positive'' effect in their ADL while using the product. While there were no significant differences in the clinical parameters of disability (EDSS scores) between the treatment groups, there was a trend of beneficial effect of GranaGard, on the verbal testing during the first 3-month period of treatment. The z score for CVLT-II, significantly increased (from 0.891 to 1.415, p=0.012, Wilcoxon rank test) at 3-months in the group of patients who were treated with GranaGard, as compared to baseline. A similar (but not statistically significant) trend was seen also in the BVMTr testing during the same 3 months-period, whereas there was no change in the SDMT. The overall average z-score of all three cognitive functions was significantly improved in the three months of Granagard treatment (-0.0077 at 3 months vs 0.462 at baseline, p=0.034, Wilcoxon rank test). During the same 3-months period there were no significant changes in the placebo-treated group. For the patients receiving GranaGard in the initial 3 months, the value of z score of CVLT-II remained high (z=1.415) also at the following three months (while

they received placebo) suggesting a longer lasting effect for at least 3 months after discontinuation of the drug.

Conclusion: This is the first study in which GranaGard, a brain targeted nano-formulation of PSO, was tested in humans. Our results in this small pilot, controlled trial provide indications that GranaGard administration to MS patients might improve/stabilize cognitive disability of these patients. Larger studies with longer duration, are needed to confirm these initial observations.

Funding: The study was supported by the Prusiner-Abramsky research grant and by the Principal Investigator's personal grants.

Introduction:

Multiple sclerosis (MS) is a progressive disease of the CNS. Inflammation affecting the myelin sheath and causing demyelination is considered as the main main disease process [1]. However axonal damage and loss are increasingly documented and their role in early stages, in disease progression and permanent disability is now well documented [1].

As a result of the widespread of the lesions MS patients present with several and different symptoms including motor, sensory visual and urinary deficits.

Cognitive impairment in MS has been neglected in the past. With the evolution of new, more effective disease modifying medications and the recognition of the role of axonal and neuronal damage, CNS atrophy and cognitive problems are increasingly highlightened as key parameters of disease activity [2].

Moreover, it is now recognized that cognitive problems are present from the early stages of the disease and play a central role in disability progression during all stages of the disease, with prevalence rates ranging from 43% to 70% [3]. MS affects various aspects of cognitive function including concentration, long term memory, information processing, executive functions, verbal learning and visual perceptional functions.

There are currently no approved medications for the treatment of cognitive symptoms in patients with multiple sclerosis, although few studies have shown some -non consistent- benefits [4-13]. Stronger evidence exists on cognitive training in patients with multiple sclerosis.

Oxidative damage is associated with aging and is widespread in the CNS in neurodegenerative diseases [14] and may therefore have a place in the management of cognitive decline in chronic CNS diseases such as MS.
Although pure natural products or plant extracts displaying antioxidant activity have shown very good results in in vitro and in vivo animal models of neurodegeneration, their clinical outcomes in human patients are still inconclusive and demonstrate limited success [15]. Specifically in Multiple sclerosis, a few antioxidants have shownv some promising effects in small studies [16-19].

Pomegranate seed oil (PSO) comprises a unique component, Punicic Acid (PA), a poly-saturated fatty acid, which is considered as one of the strongest antioxidants in nature. We have shown that PSO in the animal model of MS, EAE, reduced demyelination and oxidation of lipids in the brains of the affected animals, and improved their clinical disease course [20].

The aim of this study was to investigate the effects of GranaGard, a nano-formulation of Pomegranate seed oil (Granalix Bio Technologies LTD), comprising by 80-90% of Punicic Aci, on the cognitive function of MS patients.

METHODS

Patients

After an informed consent procedure at the inclusion visit, 30 participants were included in the study, randomized (for EDSS, age and type of MS) and divided into two groups (visit 1). Group A (n=15) were given GranaGard for the first three months, and placebo for the next three months and (Group B (n=15) received placebo for the first three months, and GranaGard for the following three months. Placebo and Granagard pills (kindly provided by Granalix Bio Technologies Ltd) were similarly shaped and colored pills containing either Granagard or soybean oil (placebo). Neither the patients, nor the involved physicians and

neuropsychologist were aware of the type of pills each patient received (the numbered pills arrived to the treating physician from the external randomization administrator). All patients received GranaGard for additional six months. GranaGard, was added to the designated chronic immunomodulatory MS treatment.

Three months (day 90 ± 5) after start of treatment, patients had their first follow-up visit (visit 2). A second evaluation (visit 3) was performed after 6 months (day-180 ± 30).

During every of the study visits, the following parameters of the disease were examined:

- Quality of life, and Fatigue by filling out SF-36 and the MFIS-5 questionnaires.
- Cognitive tests, including, PASAT-3 sec (Paced Auditory Serial Addition Test), Brief International Cognitive Assessment for Multiple Sclerosis (BICAMS), Symbol Digit Modalities Test (SDMT), California Verbal Learning Test (CVLT-II) and Brief Visuospatial Memory Test (BVMT-R).
- EDSS scoring and walking ability, by testing 25-feet walking time (7.5 m) (for MS patients with EDSS <7.0).

Criteria for inclusion:

- Men and women diagnosed with RRMS, according to the revised McDonald's [21].
- Age between 18-75.
- Expanded disability status scale (EDSS) between 1.0 and 7.5.
- No abnormal laboratory, medical or psychiatric findings.
-Ability to give informed consent.

Exclusion criteria:

- Severe neuropsychiatric syndromes or inability to perform cognitive tests and answer the questionnaires

Criteria for removal from the experiment:

• At the request of the patient or one of his legal representatives.

• If the researcher knows that continued participation in the study may endanger the patient's health.

• Occurrence of an abnormal event or the onset of a disease which in the opinion of the physician can be a problem in the continued participation of the patient in the study.
• In case of serious violation of the conditions of inclusion / exclusion.

Demographics

The patients' demographics, type of MS, EDSS score, duration of MS and immunomodulating treatment in each patient are shown in Table 1. As seen, the two randomized groups in our study were well-balanced and did not differ significantly to any of the above parameters.

Statistical analysis

We compared (paired analysis) the z scores of each of the three main cognitive tests (and the sum of all scores together) at 3 and 6 months compared to baseline values for each of the two groups. The analysis was performed by using the non-parametric Wilcoxon rank test using the SPSS software (version 26), due to the small numbers in each group. We focused on the changes at 3 months due to possible carryover effects during the second cycle of treatment. In order to reduce such distortions, we also used a linear MMRM model (Mixed-effect model for repeated measures) applied for analyzing the difference between the two treatments and adjusted for the carry-over effect (the analysis was performed by MEDSTAT company, Tel Aviv).

Results:

Adverse events

There were absolutely no adverse events reported in any of the patients in both groups during the two treatment periods of the study (data not shown).

Effects on cognitive function testing

There was a trend of beneficial effect of GranaGard, on the verbal testing during the first 3-month period of treatment. The z score for CVLT-II, significantly increased (from a mean of 0.891 to 1.415, median: 0.799 vs 1.199, p=0.012, Wilcoxon signed rank test) at 3-months in the group of patients who were treated with GranaGard, as compared to baseline. In order to reduce distortions produced by carry-over effects from the first to second cycle of the study, we also used a linear MMRM model (Mixed-effect model for repeated measures) applied for analyzing the difference between the two treatments and adjusted for the carry-over effect (analysis performed by MEDSTAT company, Tel Aviv). Using this analysis the effect of treatment with GranaGard on CVLT-II remained statistically significant at p=0.05. A similar trend (but not statistically significant either using the Wilcoxon signed rank test or the MMRM) was seen also in the BVMTr testing during the first 3 months treatment-period, whereas there was no change in the SDMT. The overall average z-score of all three cognitive functions was significantly improved in the three months of Granagard treatment (mean: 0.387 at 3 months vs -0.0077 at baseline, median: 0.509 vs -0.064, p=0.034, Wilcoxon signed rank test and p=0.09 in MMRM analysis). During the same 3-months period there were no significant changes in the placebo-treated group. For the patients receiving GranaGard in the initial 3 months, the value of z score of CVLT-II and the overall average z-score, remained high (CVLT-II mean: 1.415, median 1.698; overall mean: 0.462, median: 0.612) also at the following three months (while they received placebo) suggesting a longer lasting effect for at least 3 months after discontinuation of the drug.

Discussion

In the current small randomized placebo-controlled trial we detected a beneficial effect of the anti-oxidant Garanagard on cognition in Multiple sclerosis patients as evidenced by at least two cognitive tests that are known to be sensitive in detection of cognitive dysfunction in MS, the CVLT-II and the BVMTr. On the other hand, there was no change in SDMT, which might be explained by the lack of sensitivity of this test in detection of fast changes in cognitive functions. The overall average z-score of all three cognitive tests was significantly improved during the three months of Granagard treatment. Interestingly, the beneficial effects of Granagard-treatment on CVLT-II, persisted also during the following three months

while the patients received placebo, suggesting a longer lasting effect (for at least 3 months after discontinuation of the drug).

Cognitive impairment affects 40% to 60% of individuals with MS [4] [22] and no medication has been consistently shown to be effective for this disability. The currently available disease-modifying drugs for MS has shown some minor beneficial effects also in cognitive functions. In a metaanalysis that included any study examining change in cognitive performance, which encompassed 55 cohorts from 44 studies, disease modifying therapies showed gains in either SDMT or PASAT with a small to medium effect size [9].The differences in effects on cognition, as measured by SDMT between first-line and escalation (higher efficacy) immunotherapies, seem to be rather small [9].

Various other symptomatic medications were tried for cognition in MS. One controlled trial in mildly impaired patients with MS showed that donepezil relative to placebo led to modestly greater improvements on a test of verbal learning and memory [7]. However, a subsequent multi-center study failed to confirm this benefit as donepezil did not differ from placebo in improving a test of verbal memory or self-reported memory change [8]. Donepezil also failed to improve any of the other neuropsychological measures. Different AChEI (rivastigmine) [13] or other agents such as pemoline [6], amantadine [6], ginkgo biloba [10, 15], and amphetamine [23] have also failed to show a consistent benefit on primary cognitive outcomes.

Like all symptoms of multiple sclerosis, cognitive impairment is characterised by high variability between patients [24]. CPS, learning, and memory are most frequently involved. Deficits in executive function and visuospatial processing are also reported, but less frequently [24]. In particular, in a representative sample of 291 adult patients with any type of multiple sclerosis,4 the frequencies of impairments (varying by test) were as follows: 27–51% in CPS, 54–56% in visual memory, 29–34% in verbal memory, 15–28% in executive function, and 22% in visuospatial processing. Basic language, semantic memory, and attention span are rarely impaired (in about 10% of patients with multiple sclerosis). However, some studies suggest that semantic fluency is more often compromised than was previously thought, especially in patients older than 50 years [25, 26].

Cognitive impairment occurs in all multiple sclerosis phenotypes [27, 28]: estimates are 20–25% of patients with clinically isolated syndrome and radiologically isolated syndrome, 30–45% of patients with relapsing-remitting multiple sclerosis, and 50–75% of patients with secondary progressive multiple sclerosis. Prevalence in primary progressive disease varies greatly.

Two consensus conference initiatives recommended optimal brief cognitive assessment batteries for multiple sclerosis: the Multiple Sclerosis Outcomes Assessment Consortium (MSOAC) [29] and the Brief International Cognitive Assessment for Multiple Sclerosis (BICAMS) [30]. The MSOAC included the SDMT [31] as the sole cognitive measure in an attempt to replace the Paced Auditory Serial Addition Test (PASAT)50 as the gold standard for clinical trials, and to facilitate inclusion of cognitive measures in clinical trial design by promoting their acceptance by regulatory agencies.

By contrast, the BICAMS was an attempt to translate and validate cognitive tests that are simple to administer for the clinical care of patients with multiple sclerosis. Devoted only to cognition, BICAMS includes the SDMT, the BVMTR, and either the RAVLT or CVLT [32] [33]. The frequently used verbal memory test, the California Verbal Learning Test (CVLT) [34], also discriminates cognitive impairment in patients with multiple sclerosis from otherwise healthy controls abd is included in the BICAMS battery. An additional visual memory test called the Brief Visuospatial Memory Test Revised (BVMTR) [35] was also effectively used in MS. These memory tests (ie, RAVLT, CVLT, and BVMTR) are nearly as effective as the SDMT at distinguishing cognitive impairment in patients with multiple sclerosis from otherwise healthy individuals.

Oxidative damage is associated with aging and is widespread in the CNS in neurodegenerative diseases [14]. Free radical species mediate damage to proteins, lipids, mitochondria, and DNA and may activate the cell cycle; overwhelm endogenous antioxidant defenses in the brain; and contribute to neuronal damage [14] [36]. Observational studies suggest that an antioxidant-rich diet may reduce the risk of Alzheimer's disease [37]. However, prevention trials of combinations of antioxidant dietary supplements in elderly subjects did not influence age-associated cognitive decline [38]. Antioxidant randomized clinical trials have had mixed results [11] [39].

Although pure natural products or plant extracts displaying antioxidant activity have shown very good results in in vitro and in vivo animal models of neurodegeneration, their clinical outcomes in human patients are still inconclusive and demonstrate limited success [15].

Specifically, in Multiple sclerosis, since oxidative damage has been known to be involved in inflammatory and autoimmune-mediated tissue destruction in which, modulation of oxygen free radical production represents a new approach to the treatment of inflammatory and autoimmune diseases. Several experimental studies have been performed to see whether dietary intake of several antioxidants can prevent and or reduce the progression of EAE or not. Although a few antioxidants showed some efficacy in these studies, little information is available on the effect of treatments with such compounds in patients with MS [16-19].

Pomegranate seed oil (PSO) comprises a unique component, Punicic Acid (PA), a poly-saturated fatty acid, which is considered as one of the strongest antioxidants in nature. To increase its bioavailability and activity, PSO was prepared in oil-in-water nano-emulsions (denominated as Nano-PSO) [40]. This approach allows the distribution of the Punicic acid to organs other than the liver, and especially reach and pass the blood brain barrier (BBB) [41].
In previous studies in our center [20] PSO was administered to mice afflicted with the animal model of Multiple Sclerosis. The treatment reduced demyelination and oxidation of lipids in the brains of the affected animals, as well as improved their clinical disease course.

GranaGard, a nano-formulation of Pomegranate seed oil, comprises 80-90% of Punicic Acid and is one of the strongest natural antioxidants, has proved to prevent neuronal death in several mice models, such as EAE for MS, TgMHu2ME199K for CJD and 5XFAD for AD suggesting neuroprotective effects.

Based on the above knowledge concerning the pathogenesis of atrophy, neurodegeneration and cognitive decline in MS and the central role of oxidative stress in these processes, the beneficial effects observed in our study seem to be well explained by the amelioration of the oxidative stress that compromises neuronal functioning in chronic CNS diseases such as MS. The rather rapid effect, could be explained by the fast acting anti-oxidant effect of

Granagard which may possiibly affect the toxic milieu surrounding the partially damaged demyainated neuronal cells and axons. Interestingly this beneficial effect was continued even during the second period of 3 months in which the initially Granagard-treated patients were given only placebo. The lack of statistically significant effect on other cognitive tests, as SDMT (although there was a positive trend) could be explained by the small size of the groups tested and possible less sensitivity of SDMT for fast changes in cognitive tests. Other possibilities, such as possible differential effects of Pomegranad of distinct neuronal networks could be also possible and have to be evaluated in larger groups and longer follow up periods.

The strength of this our trial -despite its small size- is related to its randomized, double blind and crossover design allowing to detect signals/indications of short-term effects on cognition. The obvious limitations of the current study are certainly related to its small size and short duration.

In conclusion, this pilot, controlled trial, is the first study in which GranaGard, was tested in humans, providing indications that the strong anti-oxidant GranaGard may improve or stabilize cognitive functioning in MS patients. Certainly, larger studies with longer duration, are needed to confirm these initial observations.

Conflict of interest: The authors have no conflict of interest to report regarding the current study.

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CRediT author statement

Panayiota Petrou: Conceptualization, Methodology, Original draft preparation**,** Writing- Reviewing and Editing, **Ariel Ginzberg.**: Formal analysis of data, Writing, **Orli Benyamin**: Conceptualization, Methodology. **Dimitrios Karussis:** Conceptualization, Methodology, Writing- Reviewing and Editing, Original draft preparation

Figure 1

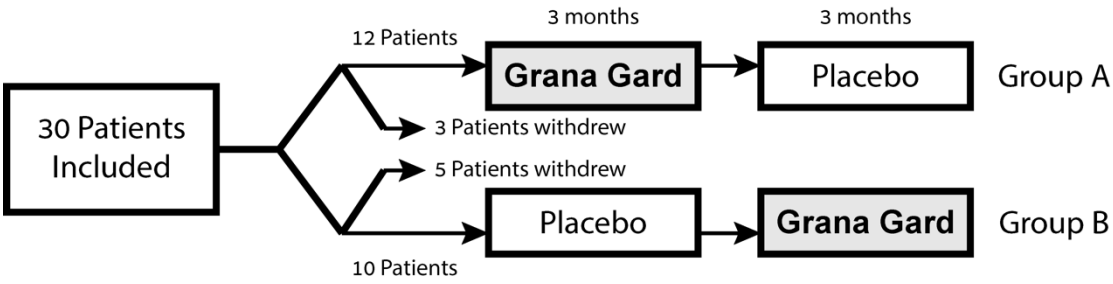


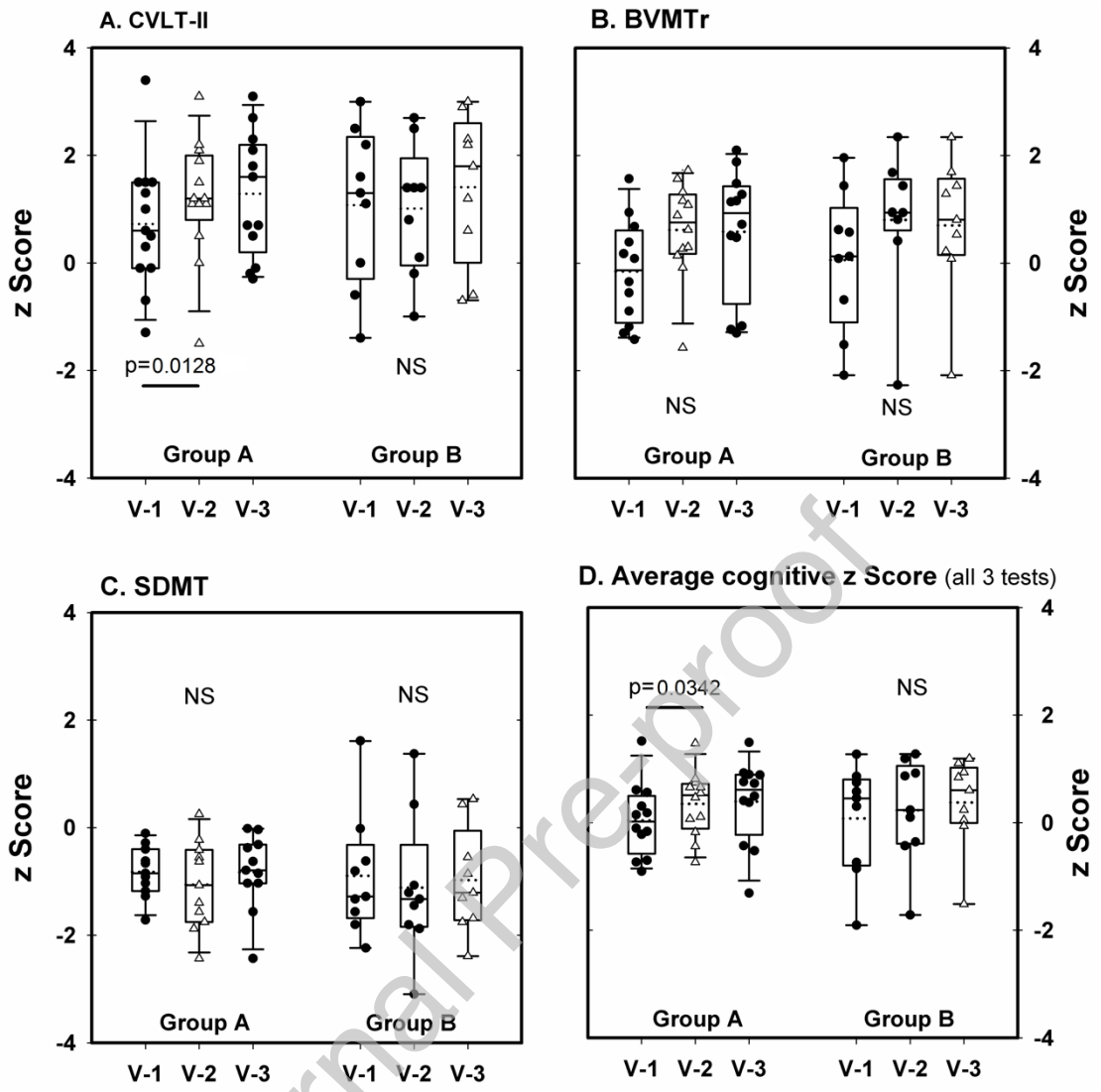
Figure 1
Study design

Table 1

Patients' Demographics

	Group A	Group B	P value
n	12	10	
F/M	7/5	7/3	0.57
Age (y): mean±SD	47.7±14.0	50.1±8.9	0.62
Onset disease (y): mean±SD	14.8±12.0	19.6±11.5	0.18
EDSS: mean±SD	4.5±1.9	5.0±1.2	0.81
MS Type:			
RRMS	9	7	
PPMS	2	1	
SPMS	1	2	
Disease Modifying Therapies			
- Ocrelizumab	4	5	
- Natalizumab	2	2	
- Dimethyl Fumarate	2	1	
- Fingolimod	1	0	
- Rituximab	1	0	
- Teriflunomide	1	0	
- Peginterferon beta-1a	1	0	
- Interferon beta-1b	0	1	

Figure 2



Legend to Figure 2: Longitudinal follow up of the changes in cognitive functions tested by 3 tests (CVLT-II, BVMTr and SDMT) during the visits 1 to 3 (ie baseline, 3 months and 6 months) and the two cycles of treatment (Group A: treated with Granagard during the first 3 months and with placebo between months 3 and 6; Group B: treated with placebo during the first 3 months and with Granagard between months 3 and 6).

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