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# Effects of DHA-phospholipids, melatonin and tryptophan supplementation on erythrocyte membrane physico-chemical properties in elderly patients suffering from mild cognitive impairment

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ABSTRACT

A randomized, double-blind placebo-controlled clinical trial was carried out to assess the efficacy of a docosahexenoic acid (DHA)-phospholipids, melatonin and tryptophan supplemented diet in improving the erythrocyte oxidative stress, membrane fluidity and membrane-bound enzyme activities of elderly subjects suffering from mild cognitive impairment (MCI). These subjects were randomly assigned to the supplement group (11 subjects, 9F and 2M; age 85.3 ± 5.3 y) or placebo group (14-matched subjects, 11F and 3M; 86.1 ± 6.5). The duration of the treatment was 12 weeks. The placebo group showed no significant changes in erythrocyte membrane composition and function. The erythrocyte membranes of the supplement group showed a significant increase in eicosapentenoic acid, docosapentenoic acid and DHA concentrations and a significant decrease in arachidonic acid, malondialdehyde and lipofuscin levels. These changes in membrane composition resulted in an increase in the unsaturation index, membrane fluidity and acetylcholine esterase activity. Moreover, a significant increase in the ratio between reduced and oxidized glutathione was observed in the erythrocyte of the supplement group.
Although this study is a preliminary investigation, we believe these findings to be of great speculative and interpretative interest to better understand the complex and multi-factorial mechanisms behind the possible links between diets, their functional components and possible molecular processes that contribute to increasing the risk of developing MCI and Alzheimer's.



## 1. Introduction

The characteristic features of Alzheimer's diseases include the presence of neurofibrillar deposits of amyloid β peptides within the brain, neuronal degeneration and cognitive losses (Hashimoto et al., 2006). A great deal of work has been performed on amyloid β peptide-induced neurotoxicity including oxidative stress (Butterfield and Lauderback, 2002), disruption of $Ca^{2+}$ homeostasis (Kawahara and Kuroda, 2001) and cholinergic signalling pathways (Kelly et al., 1996). It has been reported that amyloid β deposition is initiated as a result of direct interaction with bilayer membranes (Yamaguchi et al., 2000) and amyloid β conferring toxicity through the modulation of physico-chemical properties, in particular the fluidity of membranes, which are the first point of contact with all mechanical stresses (free radicals and peroxidation processes, inflammation, etc.) from both within and extracellular milieu. Docosahexenoic acid (Suzuki et al., 1998) and melatonin (Garcia et al., 2011) have been shown to provide a more fluid state of the bilayer membrane in a variety of cells, including neuronal cells. This fluid state improves cellular insulin resistance thus reducing the severe abnormalities in the brain glucose/energy metabolism and insulin signalling documented to play a pivotal role in Alzheimer's disease (Correia et al., 2011): in fact it has been hypothesized that this membrane fluidity-dependent "insulin-resistant brain state" forms the core of the neurodegenerative events that occur in Alzheimer's disease (Schubert et al., 2004). Neuronal membrane DHA concentration (Soderberg et al., 1991) and melatonin levels (Skene et al., 1990) decrease in Alzheimer patients thus resulting in reduced membrane fluidity (Heron et al., 1982). These results prompted us to study the effects of a diet supplemented with DHA-phospholipids, melatonin and tryptophan (the metabolic precursor of melatonin) in elderly patients suffering from mild cognitive impairment (MCI), a pathological state that normally precedes the onset of classic Alzheimer's (Gauthier et al., 2006). The supplemented diet in this randomized double-blind placebo-controlled clinical study showed a significant treatment effect for the mini-mental state examination and a positive trend for the

*Abbreviations:* AChE, acetylcholine esterase; DHA, docosahexenoic acid; EPA, eicosapentenoic acid; GSH, reduced glutathione; GSSG, oxidized glutathione; MCI, mild cognitive impairment; MDA, malondialdehyde; MMSE, mini mental state examination; PUFA, polyunsaturated fatty acid.

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semantic verbal fluency and olfactory sensitivity assessment in MCI patients (Rondanelli et al., 2012). To better understand the possible molecular mechanisms that could have lead to this clinical benefit, we assessed the supplement treatment-induced changes on erythrocyte membrane composition, pro-/anti-oxidant state and fluidity of these MCI patients.

## 2. Materials and methods

### 2.1. Subjects

The study was performed under the approval of the Ethics Committee of the Department of Internal Medicine and Medical Therapy, University of Pavia. Subjects gave their written consent to the study. The subjects were recruited from a long-term care facility in Pavia and they were enrolled after at least 3 months of institutionalization. Eligible patients were aged 70 years or older and had a diagnosis of mild cognitive impairment that was achieved according to the published criteria (Petersen et al., 1995) as follows: memory complaint referred by the subject and/or a family member; cognitive impairment in ≥1 domains (executive function, memory, language, or visuospatial); normal functional activities; and not having dementia. All patients having a Mini Mental State Examination (MMSE) score higher than 24 (Folstein et al., 1975) underwent a psychiatric evaluation after an in-depth clinical interview. Exclusion criteria were the following: (a) presence of a current comorbid psychiatric diagnosis; (b) presence of psychotic symptoms; and (c) current use of psychotropic drugs other than benzodiazepines. Moreover, subjects with a clinically uncontrolled organic disease or with clinically relevant laboratory abnormalities were excluded from the study. The ongoing pharmacological treatment at the inclusion time (such as drugs for insomnia, hypertension, diabetes, etc.) was maintained during the study. No intervention other than the administration of dietary supplement or placebo was performed for cognitive impairment.

### 2.2. Blood collection and analyses

Overnight fast blood was drawn from patients (12 h without food) in the morning at study entry and after the 12 weeks of treatment. Blood collection and handling were carried out under strictly standardized conditions and in line with manufacturer recommendations. Whole blood (EDTA as an anticoagulant) was used for hematological procedures and packed erythrocyte preparation (Cazzola et al., 2011). Packed erythrocytes were used for the measurement of reduced and oxidized glutathione levels and for membrane isolation.

Reduced and oxidized glutathione levels were measured fluorometrically according to Hissin and Hilf (1976).

Erythrocyte membranes (ghosts) were prepared as previously described (Cazzola et al., 2004).

The ghost content of phospholipids, cholesterol and lipofuscin was measured on lipid extracted in 2:1 chloroform: methanol (Merck, Darmstadt, D) containing 0.2% butylated hydroxytoluene (BHT; Sigma, Milan, Italy) as previously described (Cazzola et al., 2004).

Malondialdehyde (MDA) concentrations were determined spectrophotometrically after the reaction with thiobarbituric acid as described by Jentzsch et al. (1996). 1,1,3,3-Tetrethoxypropane (Sigma, Milan, Italy) was used for calibration and results were expressed as nmol MDA/mg protein.

Vitamin E levels were determined by measuring the ghost α-tocopherol content by HPLC (Cazzola et al., 2004).

Ghost fatty acid composition was determined using capillary gas chromatography as previously described (Cazzola et al., 2004). The degree of unsaturation of erythrocyte membrane (unsaturation index, U.I.) was calculated as the sum of each unsaturated fatty acid concentration multiplied by its double bond number and divided by the total unsaturated fatty acid concentration.

The Na, K ATPase activity was measured as the ouabain-inhibitable inorganic phosphorus released after incubation of membranes with ATP (Raccah et al., 1996), whereas the acetylcholinesterase (AChE) activity was determined according to Vander Jagt et al. (1982).

Membrane fluidity was evaluated by measuring the steady-state anisotropy (rs) of diphenylhexatriene (Molecular Probes Europe BV, Leiden, NL) as previously described (Cazzola et al., 2004).

All frozen samples were thawed only once, at the time of the assays, and, samples collected at baseline and at the end of the study from each subject were analyzed in duplicate in the same assay to eliminate inter-assay variability.

### 2.3. Study design

This is a randomized double-blind placebo-controlled clinical trial to assess the efficacy of a dietary supplement in ameliorating erythrocyte oxidative stress, membrane fluidity and membrane-bound enzyme activities (acetylcholine esterase and Na, K ATPase) in elderly subject suffering from mild cognitive impairment. The nutritional composition of 2 cps of the dietary supplement was: DHA 720 mg, EPA 286 mg, vitamin E 16 mg, soy phospholipids 160 mg (phosphatidylinositol, phosphatidylcholine and phosphatidylethanolamine), tryptophan 95 mg and melatonin 5 mg. The placebo was prepared by the manufacturer with identical capsules, using non-fish oils (without omega-3 or omega-6 fatty acids) with the appropriate colorings and additives to give the placebo capsules the same consistence and color. The products were manufactured by IBSA Farmaceutici, Lodi, Italy.

Subjects were randomized to 2 capsules, orally, once a day, 1 h before bedtime (bedtime was between 9.00 and 9.30 p.m.), or an identical placebo for 12 weeks.

Each capsule of the identical product designed for each treatment group was labelled with subject number and a code (A or B) for treatment assignment, according to a simple randomization list prepared by an independent statistician. Both preparations were identical as for aspect, smell and taste. Investigators were blinded to the randomization table, the code assignments and the procedure. At the time of enrolment, each subject was assigned a progressive subject number.

Compliance to the supplementation regimen was defined as the number of capsules actually taken by each subject divided by the number of capsules that should have been taken over the course of the study.

Patients followed a standardized diet pattern formulated by the nutritionists of the unit of dietetics and clinical nutrition based on the needs of the patients. The food intake was based on a well-balanced diet (with standard caloric (an average of 5830 kJ/day) and nutrient contents (an average/day of 15% protein, 55% carbohydrate, and 30% lipids with an average of 5.5 g weekly of DHA)) provided by the hospital kitchen. Two senior dieticians have checked daily that all the food provided to patients has been taken from them. Patients ate three meals daily, with breakfast between 07:00 and 08:00 a.m., lunch between 12:00 and 1:00 p.m., and dinner between 6:00 and 7:00 p.m.

### 2.4. Statistical analysis

Results are reported as mean ± standard deviation (SD). Baseline characteristics of study population have been checked by mean of Mann–Whitney test. The effects of treatment were analyzed by Wilcoxon's signed-rank test, p-values ≤ 0.05 were considered significant. All statistical analyses were performed by using StatistiXL software (version 1.5; StatistiXL, Western Australia).

## 3. Results

The characteristics of the study population were reported recently (Rondanelli et al., 2012) and will be summarized hereunder. Thirty-two of consecutive 200 institutionalized elderly subjects have been considered eligible for the study after neuropsychiatric evaluation. Seven