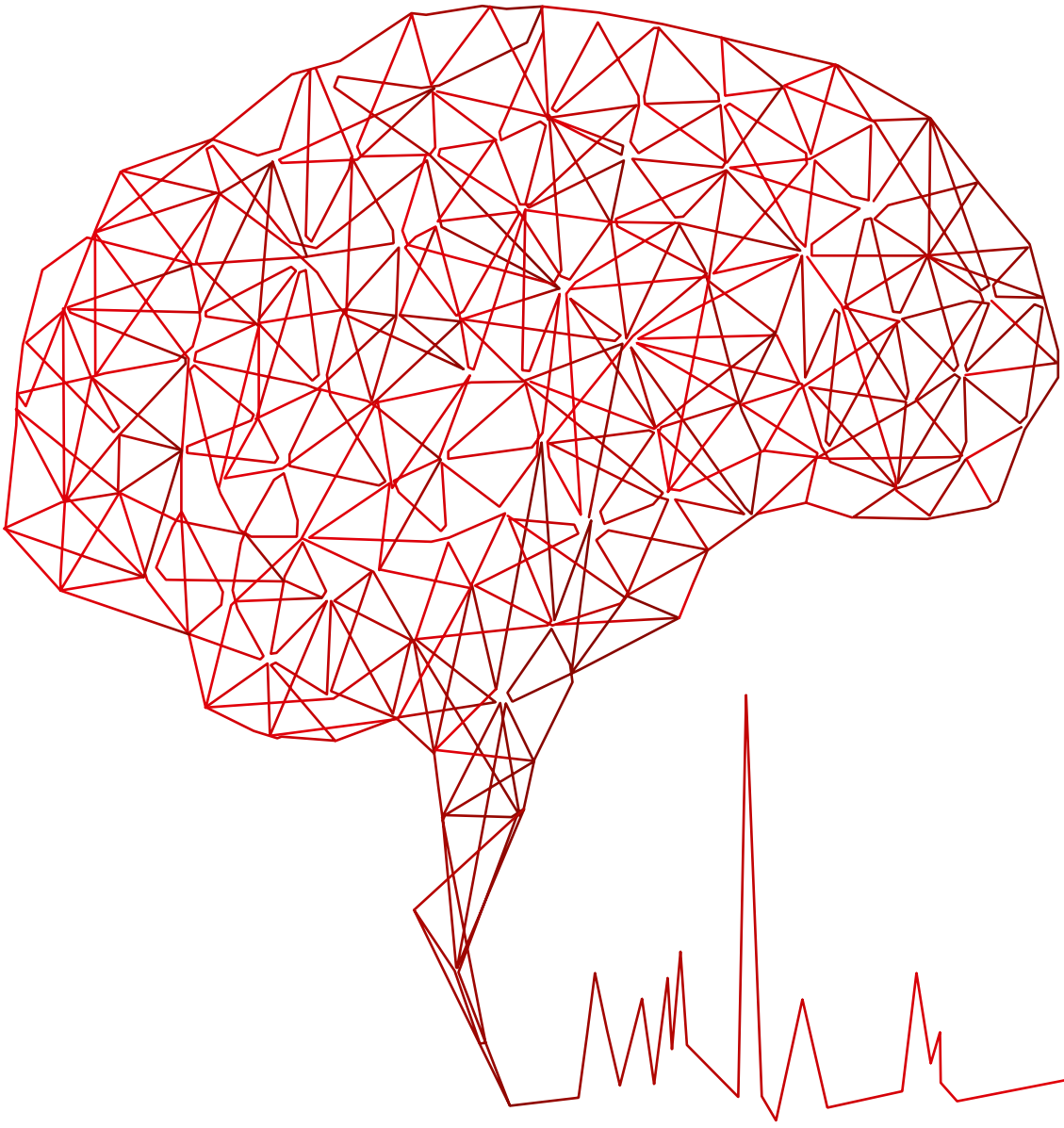

MULTI-NUTRIENT INTERVENTIONS AND BRAIN METABOLISM IN ALZHEIMER'S DISEASE: A SPECTRUM OF EFFECTS

ANNE RIJPMA

Multi-nutrient interventions and brain metabolism in Alzheimer's disease: a spectrum of effects.

Anne Rijpma

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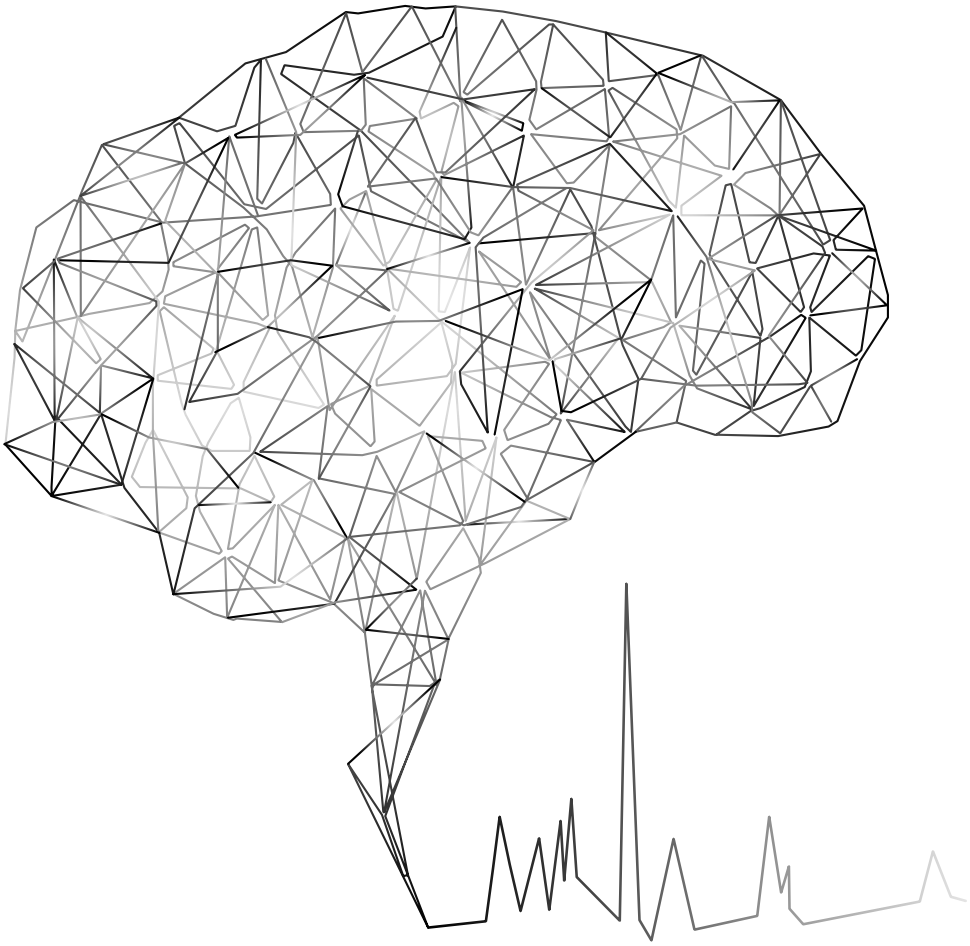
General Introduction



“Woke up lost
In a world I didn’t know
I shook it off and
I’m trying to make a go”

[...]

“All things being equal
I’d rather not forget
The things I’ve seen
And the people that I’ve met”

Eels, parallels

Alzheimer's disease

Worldwide an estimated 47 million people suffer from dementia, with about half of the cases being caused by Alzheimer's disease (AD)[1]. Advancing age is the most important risk factor for AD with an incidence of 0.4% per year in people aged 60 up to 64 years and rising to 10.5% per year in the population over 90 years of age[1].

Alzheimer's disease is a progressive neurodegenerative disorder causing cognitive decline leading to loss of independence, institutionalization and eventually death. Like any type of dementia it is characterized by memory loss and by cognitive dysfunction affecting other domains, such as planning, inhibition, language, or wayfinding. In the brain the classic pathologies in AD are progressive atrophy, amyloid plaques and neurofibrillary tangles. In addition, vascular pathology, an altered membrane composition, synaptic dysfunction and a loss of enzymes related to energy metabolism have been observed[2-4]. A wealth of modern imaging techniques are now able to demonstrate and follow alterations *in vivo*, such as glucose hypometabolism, reduced task-related activity, reduced resting default-mode activity, loss of white matter tracts, and hypoperfusion[5,6]. Although ultimately the entire brain is affected by AD, many pathologies develop in distinct regional patterns with anatomical preferences (Figure 1)[7]. Figure 2 shows several of those important brain structures, that are known for their involvement in AD. The hippocampus (HC) and adjacent cortices (medial temporal lobe) show characteristic atrophy and tangles of neurofibrillary tau. The retrosplenial cortex (RSC), part of the posterior cingulate cortex, is another region affected by atrophy and tau tangles early in the course of the disease, but this region also displays marked glucose hypometabolism. Finally, the anterior part of the cingulate cortex, anatomically and functionally part of the limbic system and the default mode network, is most likely affected by degeneration of connecting pathways from the medial temporal lobe and RSC.

Despite over a century of research, no cure for AD is currently available. Drugs have so far yielded disappointing results, with very modest efficacy and many side effects. In the past decades, several modifiable risk factors have been identified that open a window of opportunity to prevent or slow cognitive decline[8]. These mainly consist of lifestyle habits such as diet and physical, cognitive and social activity. There is strong epidemiological evidence that these factors can reduce the risk of cognitive decline and of developing dementia and/or AD[8], and may be related to the recently reported reduced incidence of dementia in high-income countries[9,10]. This indicates that these lifestyle factors may have a causative or protective role in AD and raises the question whether changing lifestyle, or lifestyle components, can slow, revert or prevent cognitive decline. In this thesis, I will focus on the role of diet, and more specifically nutritional supplements, in AD.

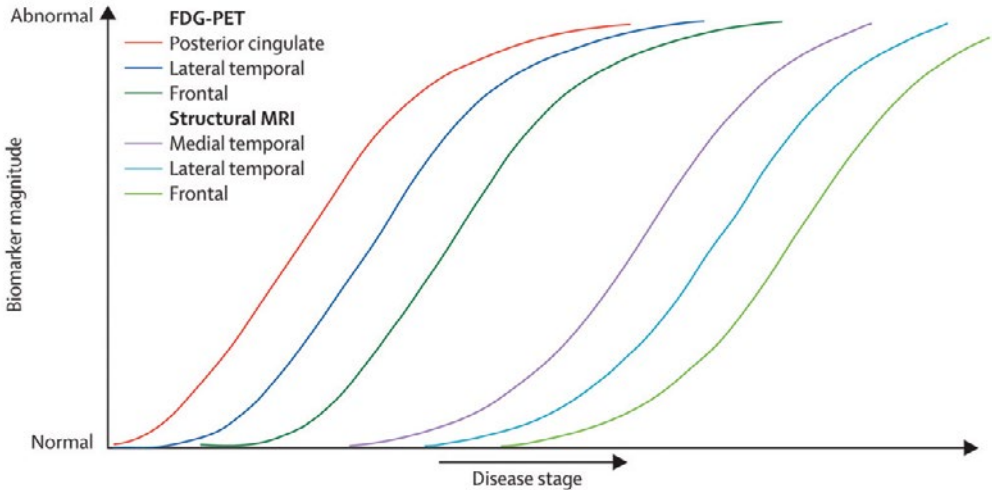


Figure 1

Anatomical imaging information. Hypothetical graph of appearance of imaging biomarker abnormalities in different brain regions. Anatomical variation exists in the time courses of biomarker abnormalities within imaging modes. For example, in FDG-PET, one would expect abnormalities to appear in the following order: precuneus/posterior cingulate, lateral temporal, and frontal lobe much later. Similarly, in structural MRI, one would expect abnormalities to appear in the following order: medial temporal, lateral temporal, and frontal lobe later. FGD-PET, fluorodeoxyglucose positron emission tomography; MRI, magnetic resonance imaging. Reprinted with permission from Jack et al. (2010)[7]; figure caption adapted for clarity.

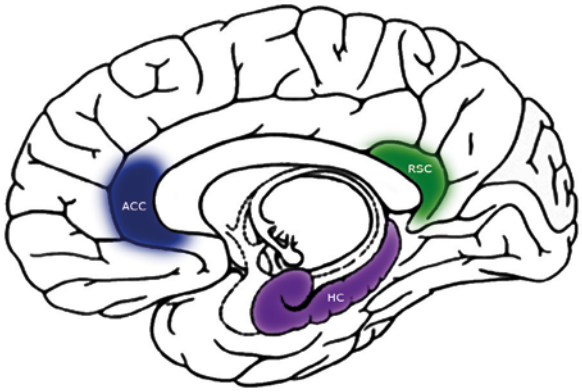


Figure 2

The main brain structures involved in Alzheimer's disease. ACC, anterior cingulate cortex; RSC, retrosplenial cortex; HC, hippocampus.

Diet as a modifiable risk factor

Diet can have a major impact on health and disease, in that it can both prevent and cause disease, but also improve or worsen recovery from (treatment of) disease. This also gives us opportunities for prevention, risk reduction and possibly even treatment of disease, including diseases that affect brain health and cognition.

Epidemiological data are in favor of the beneficial effects of diet or of specific nutrients in AD[11]. Adhering to certain diets (e.g. Mediterranean diet) or having a balanced diet in general (preventing obesity) can reduce the risk of dementia and/or AD. Regarding specific food components, one example is the intake of products high in omega-3 fatty acids (e.g. oily fish, walnuts) that lowers the incidence of dementia up to 60%[12]. Conversely, many nutrient levels are low or even deficient in patients with AD compared with the general population[13]. One can make a distinction between the effects of macronutrients (e.g. carbohydrates, proteins, and fats) and micronutrients (e.g. vitamins and minerals) on brain disease[14,15]. Macronutrients have their effects on brain health mainly through weight, muscle mass, blood pressure, cholesterol levels, and insulin functioning. For instance, obesity at midlife increases the relative risk of dementia by 42% compared with a normal body mass index[16]. However, micronutrients also have direct and specific influences on the brain. For instance, vitamins C and E, both antioxidants, can reduce oxidative stress and omega-3 fatty acids, such as docosahexaenoic acid (DHA), affect inflammatory markers and membrane fluidity[14,15]. Figure 3 shows several commonly investigated nutrients and the pathways through which they may impact on brain health and cognition. Suboptimal nutrient intake can have adverse effect on cognition and raise the risk of dementia. On the other side, heightened intake may have protective or enhancing effects on cognition. The beneficial effects of various nutrients on brain function and cognition are further explored in this research. The main focus will be on nutrients that are relevant to membrane function. Because the neural membrane is mainly composed of phospholipids, influencing phospholipid metabolism is a promising approach if membrane function is the process we aim to affect.

Brain phospholipid metabolism as a target for intervention

The brain is an organ with a very high lipid content compared with other organs. Moreover, a vast diversity exists in lipid species, which is pivotal in the various specialized membranes that exist in the nervous system[17]. The neural membrane is mainly composed of phospholipids, of which phosphatidylethanolamine (PE) and phosphatidylcholine (PC) are the most abundant[17,18]. The distribution and metabolism of these phospholipid species depend on membrane functionality and cell type. For instance, at synaptic sites the membrane is enriched with DHA-containing PE[17]. In general, neurons are also more active than glia cells in metabolizing glycerophospholipids[17], indicating a larger dependence on phospholipid metabolism. This is especially clear at the synapse, which is a site with high membrane turnover, e.g. for the recycling of synaptic vesicles. Synaptic functioning is therefore considered to be dependent on membrane phospholipid metabolism. Synaptic dysfunction is a major contributing factor to cognitive impairment in AD[19], and altered phospholipid metabolism is thought to be imperative to this deficit[18,20].

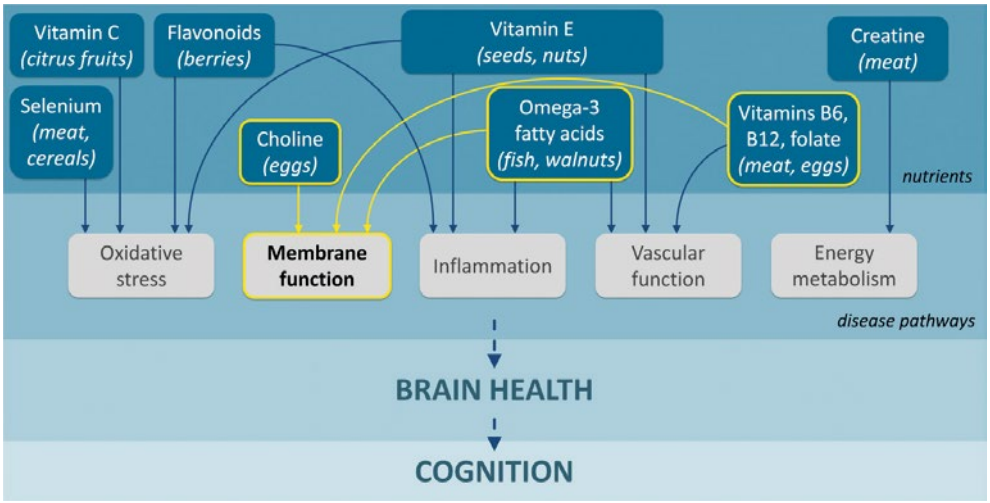


Figure 3

Overview of nutrients and the ways they may affect cognition. The research in this thesis mainly concerns nutrients that are relevant for membrane function (delineated in yellow).

The formation of PE and PC, which represent approximately 30% and 47% of neuronal membrane phospholipids[17], is mainly controlled by the uptake of nutrients, consisting of phospholipid precursors and cofactors that influence precursor uptake and metabolism, from the circulation. This phospholipid synthesis is described by the Kennedy cycle[21]. Because the enzymes in this pathway are unsaturated under normal conditions and have low affinity for their substrates, raising circulating levels of those substrates can increase the formation of PE and PC. Furthermore, increasing the availability of all precursors (i.e. uridine monophosphate (UMP), DHA, and choline) has a synergistic effect on phospholipid formation and on dendritic spine density in animal models[22]. Circulating levels of most of these rate-limiting phospholipid precursors and of the cofactors in the phospholipid synthesis pathway have been shown to be decreased in patients with AD[13,23], thereby providing a target for intervention. Furthermore, neuronal PE content in end-stage AD is lower than in brains obtained from people without neurodegenerative disease[18]. This suggests that low circulating levels of phospholipid precursors impact on phospholipid membrane composition and that these membrane alterations may be specific for AD.

Nutritional interventions that contain phospholipid precursors and co-factors for phospholipid synthesis are expected to impact on phospholipid metabolism in the brain, especially in subjects with low baseline circulating levels. Figure 4 shows the Kennedy pathway of phospholipid synthesis, including the nutrients that are known to impact on the pathway and that are obtained from the circulation. Unfortunately, it is impossible to assess the composition of the neuronal membrane by life, but phosphocholine, phosphoethanolamine, glycerophosphocholine and glycerophosphoethanolamine can be assessed *in vivo* by phosphorus magnetic resonance spectroscopy (MRS). In the next section, a brief description of this technique will be given.

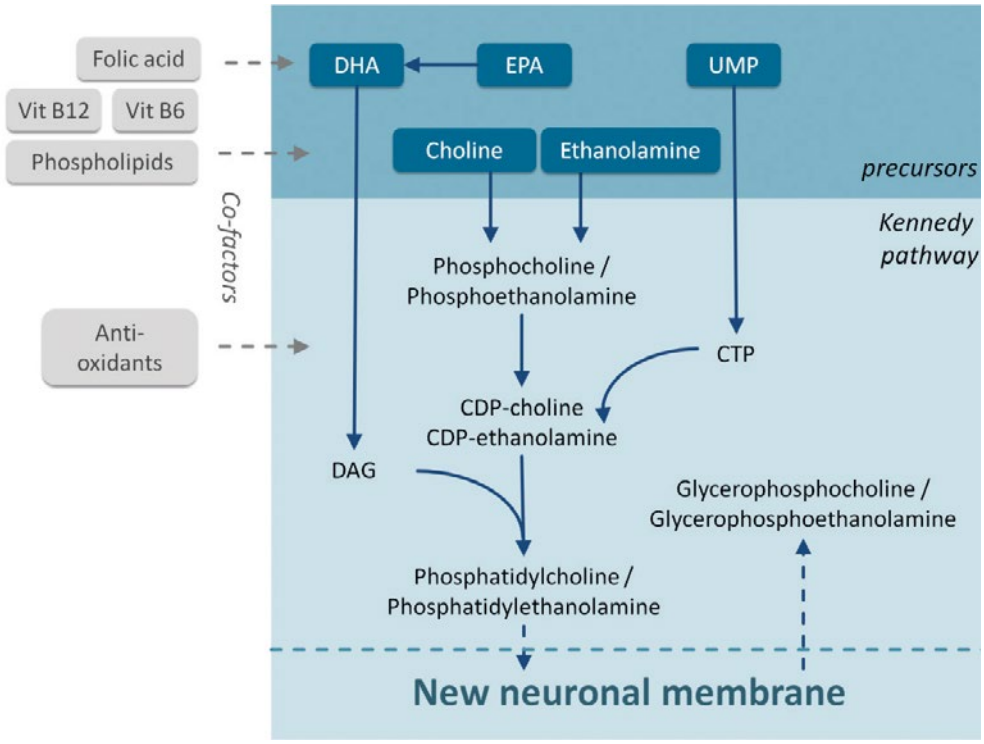


Figure 4

Kennedy pathway of phospholipid synthesis and its precursors and co-factors. CDP, cytidine diphosphate; CTP, cytidine triphosphate; DAG, diacylglycerol; DHA, doco-sahexaenoic acid; EPA, eicosapentaenoic acid; UMP, uridine monophosphate; vit, vitamin.

Techniques used in this thesis

Magnetic resonance spectroscopy

MRS allows for the *in vivo* investigation of brain metabolism and is the predominant technique used in the research in this thesis. MRS is closely related to magnetic resonance imaging (MRI), well known for its use in the clinic to make images of organs and other soft tissues. The principle behind MR is a property of atomic nuclei called spin magnetic moment or 'spin' and the use of strong magnetic fields and radiofrequency (RF) pulses to manipulate those spins[24]. Only certain nuclei have spin and are 'MR active'. Their spins can be considered as little magnets with a field that has a specific direction. Outside a magnetic field the billions of spins in the body are oriented in all directions. But in a magnetic field the spins will precess around the axis of this magnetic field with a specific frequency (ω) dependent on the strength of the magnetic field (B_0) and their intrinsic gyromagnetic ratio (γ). This frequency is called the Larmor frequency and is given by: $\omega = \gamma \cdot B_0$.

The net magnetization created by all spins is a magnetization vector in the direction of the magnetic field. After excitation of the spins by an RF pulse provided by a transmit coil, this magnetization vector is tilted in the transverse plane, perpendicular to the main magnetic field. In this plane it precesses around the direction of the main magnetic field, also with the Larmor frequency. As a result, a current is induced by this rotating net magnetization in a receiver coil. This can be compared to the rotation of a magnet in a bicycle dynamo that induces a current in a copper wire and produces light. This forms the basis of the MR signal. In MRI the signal of protons in water are used to reconstruct an image. Because the human body consists mainly of water, a strong signal can be recorded.

MRS makes use of the fact that the exact frequency at which a spin precesses is influenced by the chemical environment of the nucleus, because the electrons around the nucleus shield it from the external magnetic field[25]. The Larmor frequency is now given by $\omega = \gamma \cdot (1-\sigma) B_0$, where σ is the shielding constant, which can be different for each nucleus in a molecule. The resulting difference in frequency ω is called the chemical shift. This allows for the detection and identification of compounds (metabolites), because each compound has a unique set of signals (resonances) of nuclei with different chemical shifts. An MR spectrum consists of all signals of MR visible nuclei of compounds along a frequency axis given in parts per million (ppm) with respect to the Larmor frequency of the main magnetic field. In addition, because the integrated resonance area is directly proportional to the concentration of the nucleus and thus of the compound, quantification is possible.

MR spectra can be acquired unlocalized, localized from a single voxel or from multiple voxels using MRS imaging (MRSI). The resolution of MRSI is much smaller than the spatial resolution of conventional MRI, because the tissue concentration of metabolites is much smaller than that of water. Therefore it may be difficult to obtain signal from a single tissue type or from a small area of interest. In the brain, one can adjust or correct for this partial volume effect by taking the tissue content (gray matter, white matter, cerebrospinal fluid) obtained from structural MR images into account.

In both MR imaging and spectroscopy, proton (hydrogen, ^{1}H) is most often used as the nucleus of interest, but in principle any nucleus possessing non-zero spin has a magnetic moment and can be investigated. Nuclei besides hydrogen, so-called X-nuclei, that are biologically interesting and that possess non-zero spin include phosphorus (^{31}P), carbon (^{13}C), and sodium (^{23}Na). In this research, proton and phosphorus magnetic resonance spectroscopy (^{1}H-MRS and ^{31}P-MRS) are used. Below a brief description of both techniques will be given, in which only in vivo applications of the brain are considered.

Proton magnetic resonance spectroscopy

MR is most sensitive for hydrogen, because of its high gyromagnetic ratio and its high abundance, as many metabolites contain (multiple) protons. Therefore, ^{1}H-MRS is the most commonly used MRS technique in both biomedical research and in the clinic[26]. Furthermore, no special hardware is required in addition to conventional MRI, making ^{1}H-MRS possible on any MRI scanner. In figure 5 an example ^{1}H-MR spectrum is shown from the posterior cingulate cortex. Each metabolite results in one or more peaks at a specific frequency, with the integral of the area reflecting the concentration of that metabolite. The major brain metabolites are indicated in the figure. *N*-acetyl-aspartate (NAA) is only present in healthy neurons and is considered a marker of neural integrity. *Myo*-Inositol (mI) arises mainly from glial cells and an increase is associated with gliosis. In addition, mI functions as an osmolyte. Phosphocreatine and creatine, important in energy metabolism, cannot be separated in common ^{1}H-MRS, and are assessed together as total creatine (tCr). Finally, choline containing compounds (tCho) consist of phosphocholine, glycerophosphocholine and free choline, and are considered together as a marker of membrane turnover and phosphocholine metabolism.

In AD and its preclinical phase mild cognitive impairment (MCI) levels of NAA are reduced, indicative of loss of healthy neurons, while mI is increased, signaling increased gliosis[27-29]. Therefore, the ratio NAA/mI is decreased and is the most sensitive ^{1}H-MRS marker of AD.

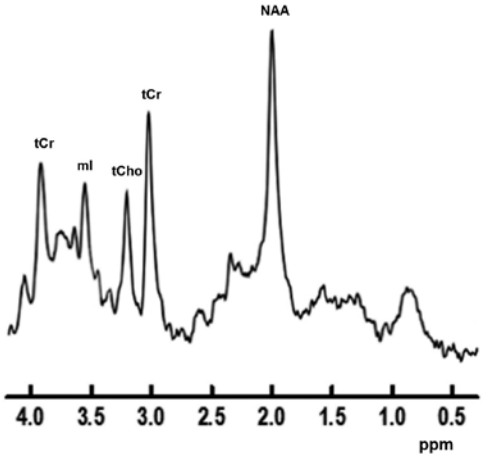


Figure 5

Proton magnetic resonance spectrum from the posterior cingulate cortex of a patient with Alzheimer's disease (67 years old) at 3 tesla. tCr, total creatine; mI, *myo*-inositol; tCho, total choline; NAA, *N*-acetyl-aspartate; ppm, parts per million.

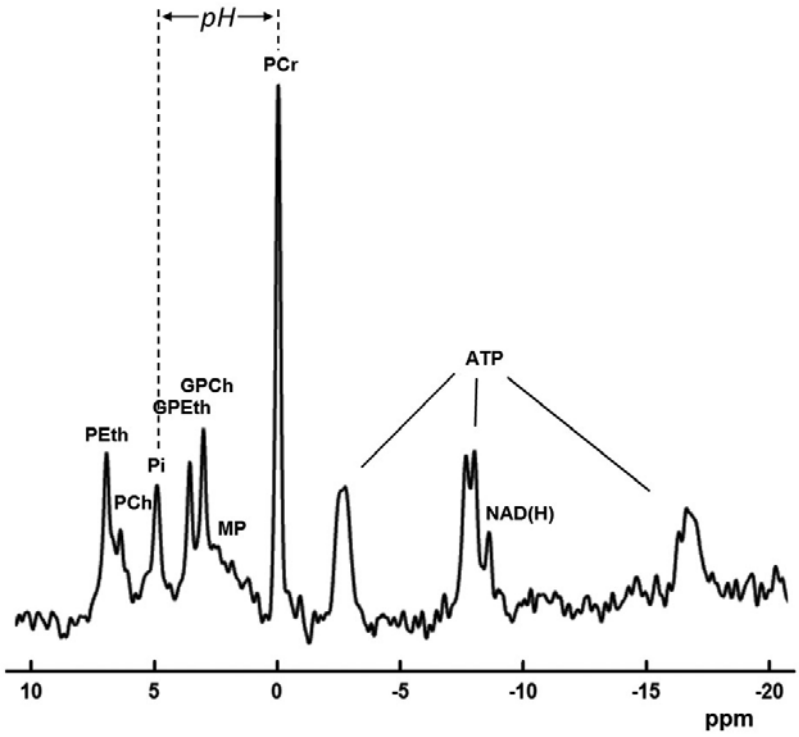


Figure 6

Phosphorus magnetic resonance spectrum from the retrosplenial cortex of a patient with Alzheimer's disease (73 years old) at 3 tesla with proton decoupling. Determination of (intracellular) tissue pH is possible from the chemical shift difference between PCr and Pi. PEth, phosphoethanolamine; PCh, phosphocholine; Pi, inorganic phosphate; GPEth, glycerophosphoethanolamine; GPCh, glycerophosphocholine; MP, membrane phospholipids; PCr, phosphocreatine; ATP, adenosine triphosphate; NAD(H), nicotinamide adenine dinucleotide; ppm, parts per million.

Phosphorus magnetic resonance spectroscopy

Although of lower sensitivity than conventional proton MRS, phosphorus can still be assessed at the clinical field strength of 3T using dedicated or double-tuned $^{1}H/^{31}P$ MR hardware. ^{31}P-MRS provides complementary information to ^{1}H-MRS on membrane turnover (phospholipid metabolism) and energy metabolism. Figure 6 shows an example ^{31}P-MR spectrum from the retrosplenial cortex. Both phospholipid building blocks (i.e. phosphomonoesters, PME) and phospholipid breakdown products (i.e. phosphodiesters, PDE)[29,30], are visible in the ^{31}P-MR spectrum. Because of the interaction of proton spins with the phosphorus spins, the ^{31}P peaks may be broadened so that some signals overlap. However, using proton-decoupling this interaction can be removed and spectral resolution is improved. As a result, spectral fitting of the PMEs phosphocholine (PCh) and phosphoethanolamine (PEth), and of the

PDEs glycerophosphocholine (GPCh) and glycerophosphoethanolamine (GPEth) separately becomes more reliable. Furthermore, key energy molecules can be assessed in the brain, such as adenosine triphosphate (ATP), phosphocreatine (PCr), inorganic phosphate (Pi) and nicotinamide adenine dinucleotide (NAD(H)). In addition, tissue pH can be determined from the chemical shift difference between the signals of Pi and PCr.

Contents of this thesis

Any drug or nutritional intervention aiming to prevent, reduce, or reverse cognitive symptoms in AD must find its way to the circulation and onwards to the brain in order to affect clinical outcome. This thesis explores the effects of nutrients in AD, with a focus on those nutrients that are involved in phospholipid metabolism, at several levels of this route.

In **chapter 2** the effect of a multi-nutrient intervention, containing phospholipid precursors and cofactors (i.e. the medical food Souvenaid®, containing the specific nutrient combination Fortasyn® Connect [Nutricia Advanced Medical Nutrition, Utrecht, the Netherlands]), on nutritional blood markers in AD is investigated. In a large sample of patients with mild to moderate AD, obtained from previously published randomized controlled trials (RCT), the influence of this multi-nutrient intervention on circulating levels of vitamins and fatty acids as well as on markers of inflammation and oxidative stress is described. Raising circulating levels of the precursors and cofactors of phospholipid formation is thought to impact on brain phospholipid metabolism and neural integrity. Although neural integrity had been extensively investigated in AD, knowledge on phospholipid metabolism was incomplete. As previous studies failed to provide conclusive evidence whether alterations are present at the early stage of AD, we investigated phospholipid metabolism in mild AD patients and healthy older persons using ^{31}P-MRS in the study described in **chapter 3**. We especially capitalized on recent technical advances in ^{31}P-MRS, such as proton-decoupling to increase spectral resolution, and elliptical weighted k-space sampling that enabled us to collect whole brain, high quality spectra, within a feasible timeframe. All measures could therefore be obtained in multiple brain regions relevant to AD, namely the anterior and posterior parts of the cingulate cortex (ACC and RSC), and the left and right hippocampus (HL and HR). Next (**chapter 4**), we investigated whether phospholipid precursors, or their metabolites, would cross the blood brain barrier to affect brain phospholipid metabolism (the *MRS AD study*). In this study, a 4-week double-blind RCT, we used ^{31}P and ^{1}H-MRS to evaluate the effect of a multi-nutrient intervention on phospholipid and energy metabolism, and measures of neural integrity and gliosis in patients with mild AD. The last step in the route from intake of nutrients to clinical efficacy is improved cognition or functional outcome. This was the focus of **chapter 5**, a systematic review in which the added benefit of nutrition to cholinesterase inhibitor therapy in AD was investigated. In **chapter 6**, the results of this thesis are summarized. Finally, in **chapter 7** the main findings and their implications for future research are discussed.

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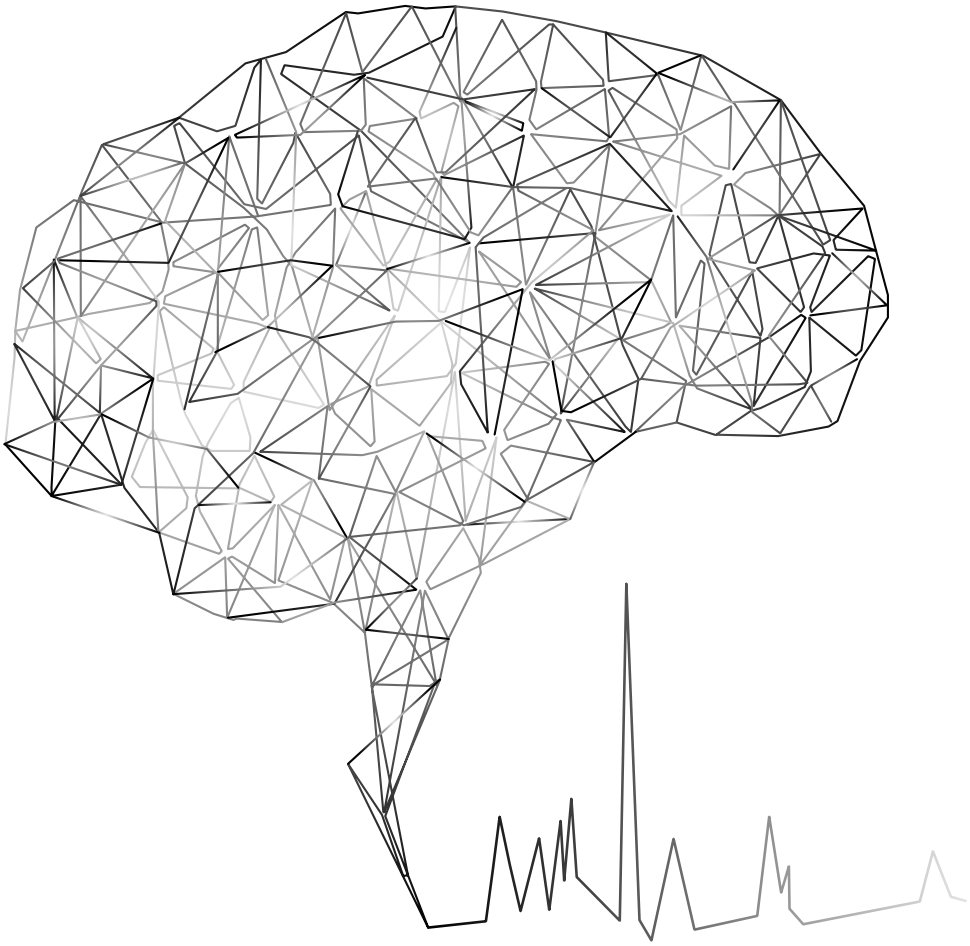
2

Effects of Souvenaid on plasma micronutrient levels and fatty acid profiles in mild and mild-to-moderate Alzheimer's disease



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Abstract

INTRODUCTION: Circulating levels of uridine, selenium, vitamins B12, E and C, folate, docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) have been shown to be lower in patients with Alzheimer's disease (AD) than in healthy individuals. These low levels may affect disease pathways involved in synapse formation and neural functioning. Here, we investigated whether, and to what extent, circulating levels of micronutrients and fatty acids can be affected by oral supplementation with Souvenaid (containing a specific nutrient combination), using data derived from three randomized clinical trials (RCT) and an open-label extension (OLE) study with follow-up data from 12 to 48 weeks.

METHODS: Subjects with mild (RCT1, RCT2) or mild-to-moderate AD (RCT3) received active or control product once daily for 12–24 weeks or active product during the 24-week OLE following RCT2 ($n = 212–527$). Measurements included plasma levels of B vitamins, choline, vitamin E, selenium, uridine and homocysteine and proportions of DHA, EPA and total n-3 long-chain polyunsaturated fatty acids in plasma and erythrocytes. Between-group comparisons were made using t tests or non-parametric alternatives.

RESULTS: We found that 12–24-week active product intake increased plasma and/or erythrocyte micronutrients: uridine; choline; selenium; folate; vitamins B6, B12 and E; and fatty acid levels of DHA and EPA (all $p < 0.001$). In the OLE study, similar levels were reached in former control product / initial active product users, whereas 24-week continued active product intake showed no suggestion of a further increase in nutrient levels.

CONCLUSIONS**:** These data show that circulating levels of nutrients known to be decreased in the AD population can be increased in patients with mild and mild-to-moderate AD by 24–48-week oral supplementation with Souvenaid. In addition, to our knowledge, this is the first report of the effects of sustained dietary intake of uridine monophosphate on plasma uridine levels in humans. Uptake of nutrients is observed within 6 weeks, and a plateau phase is reached for most nutrients during prolonged intake, thus increasing the availability of precursors and cofactors in the circulation that may be used for the formation and function of neuronal membranes and synapses in the brain.

Introduction

Several disease pathways and risk factors for Alzheimer's disease (AD) are hypothesized to be affected by nutritional factors, such as reduced neuronal membrane integrity and function, and phospholipid metabolism[1-3]. Correspondingly, epidemiological studies have repeatedly shown the protective and/or risk reducing effects of nutritional intake on AD[4-6]. In addition, patients with AD are frequently reported to have lower plasma levels of certain nutrients than healthy controls[7-10]. Data derived from our own studies have shown lower plasma levels of uridine, selenium, and docosahexaenoic acid (DHA) in patients with mild AD than in healthy age matched controls[10]. A recent meta-analysis showed lower levels in folate, and vitamins A, B12, C, and E in patients with AD than in healthy controls[7]. Together, these studies suggest a connection between nutrient status and AD. Counteracting any nutritional deficiencies may therefore have a beneficial effect on patients with AD.

The medical food Souvenaid, which contains the specific nutrient combination Fortasyn Connect (both products of Nutricia Advanced Medical Nutrition, Utrecht, the Netherlands), has been designed to address the distinct nutritional needs of patients with AD and thereby ameliorate synapse loss and synaptic dysfunction in AD. The medical food was developed to increase brain levels of specific nutrients to support the process of neuronal membrane formation[11]. In turn, increased brain nutrient levels can stimulate synapse formation to compensate for synapse loss in AD[12,13]. A prerequisite for potentially raising brain nutrient levels is that the intervention will indeed raise circulating nutrient levels, thus increasing their availability for neuronal membrane formation.

A number of clinical trials have been performed to investigate the effect of this specific nutrient combination on cognitive function in patients with mild AD[14,15] (referred to hereinafter as RCT1 and RCT2, respectively) and those with mild-to-moderate AD[16] (referred to hereinafter as RCT3). One open-label extension (OLE) study (extension of RCT2) has been performed with safety as the primary endpoint and memory as an exploratory endpoint. Both trials in drug-naïve patients with mild AD showed improvement in memory domain scores after 12 weeks (RCT1) and 24 weeks (RCT1 and RCT2)[14,15,17]. The OLE showed that use of this specific nutrient combination for up to 48 weeks was well tolerated. Furthermore, a significant increase in the exploratory memory outcome was observed in both the active-active and control-active-groups from 24 to 48 weeks of use[18]. In the clinical trial in patients with mild-to-moderate AD and on AD medication, no change in cognitive function was found after 24 weeks (RCT3) [16].

Blood samples were taken at baseline and at the end of the study in all RCTs. Here, we investigate whether, and to what extent, circulating levels of micronutrients and fatty acids, among which several are known to be decreased in the AD population, are affected by 12-48-week oral supplementation with Souvenaid, in patients with mild and mild-to-moderate AD.

Methods

Three double-blind, multicenter, controlled RCTs (Souvenir [RCT1], Souvenir II [RCT2] and S-Connect [RCT3]) were performed between 2006 and 2011, to evaluate the effects of the medical food Souvenaid on cognition and memory performance in patients with AD[14-16]. Subjects who completed the 12-week intervention of RCT1 were invited to participate in a 12-week double-blind extension period. In addition, an RCT2 OLE study was performed between 2010 and 2012 to evaluate longer-term safety of and compliance with Souvenaid[19]. Here we present the results of the analyses of secondary and exploratory plasma micronutrient parameters and fatty acid profiles in plasma, plasma phospholipids and erythrocyte membranes. Some results have been reported previously in publications describing the results of the RCTs, mainly as compliance markers[14-16,19]. All trials were registered in the Dutch Trial Register (Souvenir: NTR702; Souvenir II: NTR1975; S-Connect: NTR1683; and OLE: NTR2571).

Study population

The study population and methodology of the studies have been described in detail previously[14-16,19]. Briefly, all studies included men and women ≥ 50 years of age who were diagnosed with probable AD according to the National Institute of Neurological and Communicative Disorders and Stroke-Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA) criteria [20] and were either in the mild stage of AD as defined by Mini Mental State Examination (MMSE) scores of 20-26 (RCT1) or ≥ 20 (RCT2), or in the mild to moderate stage of AD, defined as an MMSE score of 14-24 inclusive (RCT3). Subjects in RCT1 and RCT2 had to be drug-free for AD medication, whereas subjects in RCT3 were on a stable dose of approved AD medication. Subjects were not allowed to use, within 1–2 months before study participation and during the study, fatty acid containing supplements (RCT1, RCT2, and RCT3), consume oily fish more than twice per week (RCT 2 and RCT3), use vitamins B, C and/or E >200% (RCT1 and RCT2) or >100% (RCT3) of recommended dietary allowance (RDA)[21,22]; or to use high-energy and/or high-protein nutritional supplements and/or medical foods (RCT2 and RCT3). At the end of RCT2, all subjects who completed the study were invited to participate in the OLE. Eligibility criteria for the OLE allowed patients to use AD medication and nutritional supplements.

Study procedures

All subjects were randomly assigned to receive the active or control product once-daily during 12 weeks (RCT1) or 24 weeks (RCT1 extension, RCT2, RCT3), whereas during the OLE study, all subjects received the active product. The active product (Souvenaid) contains the specific nutrient combination Fortasyn Connect (Table 1). The control product is iso-caloric and similar in appearance and flavor to the active product, but without Fortasyn Connect. Both study products were available in the form of a 125 ml drink (125 kcal) in vanilla or strawberry flavors (RCT2, RCT3 and OLE) and peach orange or cappuccino flavor (RCT1).

Table 1 Nutritional composition of the study products; amount per daily dose (125 mL)

	Control	Active
Energy	125 kcal	125 kcal
Protein	3.8 g	3.8 g
Carbohydrate	16.5 g	16.5 g
Fat	4.9 g	4.9 g
EPA	0	300 mg
DHA	0	1200 mg
Phospholipids	0	106 mg
Choline	0	400 mg
UMP	0	625 mg
Vitamin E (alpha-TE)	0	40 mg
Vitamin C	0	80 mg
Selenium	0	60 μg
Vitamin B12	0	3 μg
Vitamin B6	0	1 mg
Folic acid	0	400 μg

EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; UMP, uridine monophosphate; TE, tocopherol equivalents.

Study staff, subjects and caregivers were blinded to each subject's randomized study group allocation throughout all studies, including the extension of RCT1 and the OLE (i.e. blinding for group allocation during RCT2 continued).

Outcome parameters for the current analyses were assessed at baseline and, depending on the nutrient, week 24 of RCT1, RCT2 and RCT3 and at week 24 (presented as week 48) of the OLE. The screening/baseline visit for the OLE was combined with the final visit (week 24) of RCT2. In addition, homocysteine (Hcy), vitamin E, uric acid and percentages of DHA, eicosapentaenoic acid (EPA) and docosapentaenoic acid (DPA) of total fatty acids in erythrocyte membrane also were assessed at week 6 and week 12 of RCT1. These are not statistically analyzed in the present article. However, data on percentage DHA of total fatty acids in erythrocyte membrane is shown in Figure 1.

Written informed consent was obtained from subjects and their caregivers before study participation. The ethics committee of each participating study center in each study reviewed and approved the protocol (see Additional file 1). The studies were conducted in accordance with the Declaration of Helsinki, the International Conference on Harmonization Guidelines for Good Clinical Practice as appropriate for nutritional products and the local laws and regulations of the country in which the research was conducted.

Study parameters

Fasting (RCT1) and non-fasting (RCT2, RCT3, and OLE) venous blood samples were taken to determine plasma levels of folate, Hc), vitamins B6 and B12, choline, vitamins D and E, selenium, uridine and fatty acids (including DHA, EPA, DPA and total n-3 long-chain polyunsaturated fatty acids [n-3 LC-PUFA]). In addition, erythrocytes were collected to determine fatty acid (including DHA, EPA, DPA and n-3 LC-PUFA) levels in the erythrocyte membrane. In RCT1, the results in plasma vitamin C analyses varied greatly, preventing meaningful interpretation. For that reason, in RCT2 and RCT3, vitamin C levels were not analysed. In RCT1, venous blood samples also were taken to determine plasma levels of uracil, uric acid, cytidine, malondialdehyde (MDA), (pre)albumin, C-reactive protein (CRP), interleukin (IL)-1β, IL-6, IL-10 and 8-isoprostane.

Parameters were assessed in the intention-to-treat (ITT) population or in subgroups, based on the availability of blood samples. The actual number of analyzed samples is indicated in the tables and Figure 1 for each parameter.

Information on preexisting and new use of medication and nutritional supplements was collected throughout the studies.

Biochemical analyses

Blood was collected in tubes containing ethylenediaminetetraacetic acid. All samples were centrifuged (1300 g, 15 min, 4°C), and plasma and erythrocyte aliquots were stored at -70 °C / -80 °C (RCT1, RCT2 and OLE) or at least -20 °C (RCT3) until analysis at a central laboratory. For RCT2 and the OLE, all baseline and 24-week samples were analyzed together as part of RCT2, whereas all 48-week samples were analyzed at the end of the OLE.

Plasma folate and vitamin B12 levels were determined using a competitive protein binding ligand assay. Plasma B6 levels were measured by performing high-performance liquid chromatography (HPLC). HPLC electrochemical detection of plasma-free choline was performed according to a method adapted from one previously described by Fossati *et al.*[23] and as reported previously[24]. Plasma albumin was determined using a colorimetric kit, plasma selenium levels were measured using graphite furnace atomic absorption spectrometry and plasma pre-albumin and CRP levels were assessed using turbimetric assays. A microparticle chemiluminescent microparticle immunoassay (ARCHITECT assay; Abbott Diagnostics, Lake Forest, IL, USA) was used to determine plasma vitamin D (total 25-hydroxyvitamin D) levels. Plasma vitamin E levels were determined by performing HPLC using fluorometric properties for detection of α-tocopherol by comparison with standard solutions[25]. For the determination of plasma Hcy levels, thiol amino acids (free and protein-bound) were reduced with tri-n-butylphosphine. After precipitation with trichloroacetic acid, thiol groups were derivatized with ammonium 7-fluorobenzo-2-oxa-1,3-diazole-4-sulfonate, followed by separation using HPLC with a fluorescence detector[26,27]. Determination of MDA was based on the thiobarbituric acid and reversed-phase HPLC

separation with fluorescence detection[28]. To determine plasma uridine, uracil and cytidine levels, perchloric acid was added to the sample. Uridine, uracil and cytidine were extracted by vortexing the solution, followed by separation from other nucleotides/nucleosides using reversed-phase HPLC[29]. The compounds were quantified by measuring its absorbance compared with a standard. Uric acid levels in plasma were determined using an enzymatic assay. Plasma levels of cytokines (IL-1β, IL-6 and IL-10) and free 8-isoprostane were measured using, respectively, a commercial, custom-made human Bio-Plex cytokine bead-based immunoassay (Bio-Rad Laboratories, Hercules, CA, USA) and a commercially available enzyme immunoassay (Cayman Chemical, Ann Arbor, MI, USA) according to the manufacturers' protocol. The fatty acid composition of the total lipid fraction in plasma and erythrocytes was analyzed qualitatively on a gas chromatograph after extraction of the lipids from the plasma and/or erythrocytes and a methylation step[30-33].

Statistical analyses

Analyses were performed on the ITT population for each study or on subgroups, based on the availability of samples. Changes in outcome parameters over time were compared between groups using an independent samples t-test and/or within groups using a paired t-test. Non-parametric alternatives (Mann-Whitney U test and Wilcoxon signed-rank test) were used for non-normal distributions. For RCT2 and the OLE, sensitivity analyses were performed for vitamin B6, vitamin B12, folate, choline and Hcy excluding two patients in the control-active group because of their recent use of vitamin B12 injections prior to baseline, which might have interfered with plasma levels of these parameters. In addition, the impact of sex and age as covariates and as modifiers of the intervention effect on the laboratory parameters of RCT2 was tested using analysis of covariance (ANCOVA) with change from baseline as a dependent variable and baseline as an additional covariate. For these analyses, several parameters were log transformed to get a distribution closer to the normal distribution.

Statistical analyses were performed using SAS® software (SAS Enterprise Guide 4.3 for Windows, SAS Institute, Cary, NC, USA). Data are presented as means ± standard deviation (SD) unless stated otherwise. OLE data are presented according to the intervention received during the double-blind study period of RCT2 (i.e. control-active and active-active). Statistical significance was set at $p<0.05$ and was not corrected for multiple testing.

Results

The baseline characteristics of the total study population (RCT1: n=212, RCT2: n=259, RCT3: n=527 and OLE: n=201), are summarized in Table 2. By definition, all studies included a mild or mild-to-moderate AD population (mean MMSE scores of 23.9 [RCT1], 25.0 [RCT2], 19.5 [RCT3] and 25.1 [OLE]), and all subjects were aged 50 years or older (range: 50 - 95 years; mean age 73.7 years [RCT1], 73.8 years [RCT2], 76.7 years [RCT3] and 74.2 years [OLE] years).

Table 2 Baseline demographics and characteristics of the ITT study populations.

	RCT 1		**RCT 2**		**OLE**		**RCT 3**	
	Control (*n*=106)	Active (*n*=106)	Control (*n*=129)	Active (*n*=130)	Control-Active (*n*=104)	Active-Active (*n*=97)	Control (*n*=262)	Active (*n*=265)
Male, *n* (%)	52 (49.1)	54 (50.9)	64 (49.6)	68 (52.3)	52 (50.0)	51 (52.6)	127 (48.5)	126 (47.5)
Age, years	73.3 (7.8)	74.1 (7.2)	73.2 (8.4)	74.4 (6.9)	73.9 (8.3)	74.5 (6.8)	76.9 (8.2)	76.6 (8.2)
BMI, kg/m^2	26.2 (3.5)	26.2 (4.8)	26.7 (4.2)	26.1 (4.1)	27.3 (4.2)	26.9 (4.2)	26.6 (4.6)	26.2 (4.5)
Years of education beyond primary school	6.0 (4.0)	5.5 (3.9)	6.0 [0.0-19.0]	6.0 [0.0-20.0]	6.7 (4.7)	6.2 (4.8)	6.4 (3.5)	6.7 (3.6)
Duration of AD since diagnosis, months	32 [0-1036]*	30 [-18 1932]*	2 [0-88]	1 [0-70]	2.0 [0.0-88.0]	1.0 [0.0-70.0]	35 (30)	33 (25)
Duration of AD medication use, months	n/a	n/a	n/a	n/a	n/a	n/a	31 (29)	29 (23)
Nutritional supplement use, *n* (%)[#]								
Vitamins	6 (5.4)	4 (3.5)	12 (9.3)	11 (8.5)	7 (6.7)	8 (8.2)	104 (40.0)	112 (42.4)
Mineral supplements	9 (8.0)	15 (13.3)	12 (9.3)	7 (5.4)	10 (9.6)	4 (4.1)	50 (19.2)	46 (17.4)
General nutrients	11 (9.8)	10 (8.8)	6 (4.7)	8 (6.2)	6 (5.8)	5 (5.2)	29 (11.2)	21 (8.0)
MMSE, total score	24.0 (2.5)	23.8 (2.7)	25.0 (2.8)	24.9 (2.9)	25.1 (3.4)	25.1 (3.3)	19.4 (3.0)	19.5 (3.2)
ApoE ε4 carrier, *n* (%)								
No	-	-	58 (49.2)	62 (51.2)	46 (48.9)	41 (44.1)	84 (42.0)	87 (39.2)
Yes	-	-	60 (50.8)	59 (48.8)	48 (51.1)	52 (55.9)	116 (58.0)	135 (60.8)
Unknown	-	-	11	9	10	4	62	43

Data are mean (standard deviation) or median [range], unless indicated otherwise. -, not done. n/a, not applicable.

* In days instead of months. The value of -18 days represents a protocol deviation; the subject was diagnosed 18 days after baseline assessment.

[#] Defined as the number and percentage of subjects using at least one nutritional supplement in the all-subjects-treated population.

There were no significant or relevant between-group differences in use of nutritional supplements during the studies, except for the use of vitamin C in RCT3, which was significantly higher in the active group versus the control group (2.3% [n=6] versus 0% [n=0], p=0.030). In RCT3, a large proportion (41.2%) of subjects used (multi-)vitamins.

Plasma micronutrients and fatty acids available in Fortasyn Connect

Descriptive statistics for plasma micronutrients and erythrocyte fatty acids available in Fortasyn Connect are presented in Table 3. Results for plasma vitamin E and erythrocyte DHA and EPA have been published in part before[14-16,19].

Plasma levels of uridine, choline, folate, vitamins B6 and B12, selenium and vitamin E were all significantly increased in the active vs. control group from baseline to Week 24 in RCT1, RCT2 and RCT3 (all: $p<0.001$, except for uridine in RCT1 [$p=0.044$], Mann-Whitney *U* test) (Table 3). During the OLE, plasma levels of these parameters were significantly increased in the control-active group from week 24 to week 48 (i.e. after switching to the active product upon entry into the OLE study) (all: $p<0.001$, Wilcoxon signed-rank test). In addition, plasma levels remained consistently elevated in the active-active group during the OLE, except for plasma levels of uridine, vitamin B12 and selenium, which significantly decreased in the active-active group from week 24 to week 48 (Table 3). Despite the latter, however, there was a significant overall increase in plasma uridine and selenium, but not vitamin B12, from baseline to week 48 in the active-active group ($p<0.001$, Wilcoxon signed-rank test).

In line with the above described data, the percentages DHA and EPA of total fatty acids in both plasma and the erythrocyte membrane also were significantly increased in the active vs. control group from baseline to Week 24 in RCT1, RCT2 and RCT3 ($p<0.001$, t-test [RCT 1] or Mann-Whitney *U* test [RCT2 and RCT3]) (Table 3, Figure 1). During the OLE, levels remained consistently elevated in the active-active group and significantly increased in the control-active group from week 24 to week 48 ($p<0.001$, Wilcoxon signed-rank test) (Table 3, Figure 1). The results for n-3 LC-PUFA in plasma and erythrocytes in RCT2, RCT3 and OLE, were all in line with the results for the individual fatty acids DHA and EPA.

Comparable results were obtained from the sensitivity analyses of plasma choline, folate and vitamins B6 and B12.

A modifying intervention effect of sex on log-transformed percentage of EPA in erythrocyte membrane was found in RCT2 ($p=0.021$, ANCOVA). Post-hoc analyses revealed significantly increased percentages of EPA in the active versus control group from baseline to week 24 for both men and women, but the effect was larger in women than in men (treatment effects of 0.446 for women [$p<0.001$, *t* test] and 0.343 for men [$p<0.001$, *t* test]). No other effects of sex were found. Age was neither a significant covariate nor a significant intervention modifier for any of the micronutrients or fatty acids in RCT2.

Table 3 Descriptive statistics for plasma micronutrients, erythrocyte fatty acids, and homocysteine following Fortasyn Connect supplementation.

	Control		**Active**		***P-value ****
	Baseline	End of study	Baseline	End of study	
Uridine (μM)					
RCT 1 (0-24wk)	3.91 [1.74, 7.06] (72)	4.04 [1.16, 6.72] (68)	3.99 [1.01, 7.67] (77)	4.63 [1.47, 25.94] (72)	*0.044*
RCT 2 (0-24wk)	3.5 [0.8, 10.6] (129)	3.5 [0.6, 17.3] (119)	3.6 [0.5, 10.3] (128)	8.6 [1.8, 34.9] (116)	*<0.001*
OLE (24-48wk)			3.5 [0.6, 17.3] (103) 8.8 [1.8, 28.9] (96)	6.6 [1.6, 22.0] (95) 6.6 [1.4, 22.4] (85)	*<0.001 (C-A)* *<0.001 (A-A)*
RCT 3 (0-24wk)	3.6 [0.4, 7.7] (248)	3.2 [0.6, 25.7] (230)	3.6 [1.1, 28.8] (253)	7.4 [1.5, 32.0] (237)	*<0.001*
Choline (μM)					
RCT 1 (0-24wk)	8.54 [4.30, 19.90] (72)	8.59 [5.44, 16.00] (67)	9.64 [5.21, 22.50] (75)	11.30 [5.92, 28.10] (73)	*<0.001*
RCT 2 (0-24wk)	9.4 [4.4, 18.2] (128)	8.7 [4.5, 18.6] (117)	9.2 [4.5, 18.1] (128)	13.3 [5.8, 28.8] (116)	*<0.001*
OLE (24-48wk)			8.5 [4.5, 18.6] (101) 12.8 [5.8, 28.8] (96)	14.0 [3.6, 29.4] (91) 14.6 [7.4, 29.2]	*<0.001 (C-A)* *0.149 (A-A)*
Erythrocyte DHA (%)					
RCT 1 (0-24wk)	3.6 [0.7, 7.2] (104)	3.6 [2.0-6.9] (74)	3.6 [2.1, 6.5] (103)	7.0 [1.4-9.2] (73)	*<0.001* #
RCT 2 (0-24wk)	3.1 [0.0, 5.6] (128)	3.2 [0.7, 6.8] (119)	3.0 [0.3, 5.9] (129)	6.7 [1.3, 8.7] (114)	*<0.001*
OLE (24-48wk)			3.4 [0.7, 5.8] (103) 6.7 [1.3, 8.7] (94)	6.9 [0.4, 9.1] (93) 6.8 [1.1, 10.8] (87)	*<0.001 (C-A)* *0.853 (A-A)*
RCT 3 (0-24wk)	2.4 [0.0, 6.5] (257)	2.4 [0.2, 4.9] (232)	2.4 [0.0, 8.1] (259)	6.6 [0.9, 10.1] (239)	*<0.001*
Erythrocyte EPA (%)					
RCT 1 (0-24wk)	0.9 [0.1, 3.4] (104)	0.9 [0.1, 3.6] (74)	1.0 [0.1, 2.8] (103)	1.8 [0.0, 3.1] (73)	*<0.001* #
RCT 2 (0-24wk)	0.8 [0.0, 3.3] (128)	0.8 [0.2, 2.7] (119)	0.8 [0.0, 2.9] (129)	1.6 [0.3, 4.0] (114)	*<0.001*
OLE (24-48wk)			0.8 [0.3, 2.7] (103) 1.6 [0.6, 4.0] (94)	1.7 [0.1, 4.8] (93) 1.6 [0.5, 3.6] (87)	*<0.001 (C-A)* *0.730 (A-A)*
RCT 3 (0-24wk)	0.4 [0.0, 3.6] (257)	0.5 [0.0, 2.0] (232)	0.4 [0.0, 2.6] (259)	1.2 [0.0, 4.3] (239)	*<0.001*

Table 3 *(continued)*

	Control		**Active**		***P-value ****
	Baseline	End of study	Baseline	End of study	
Plasma DHA (%)					
RCT 1 (0-24wk)	1.8 [0.8, 4.9](91)	1.9 [1.0, 5.4](54)	1.9 [0.8, 6.2](91)	3.8 [1.2, 6.6](66)	*<0.001 #*
RCT 2 (0-24wk)	1.7 [0.7, 3.4] (129)	1.6 [0.6, 3.4] (119)	1.7 [0.7, 4.3] (129)	4.7 [1.3, 7.7] (115)	*<0.001*
OLE (24-48wk)			1.6 [0.8, 3.4] (103) 4.7 [1.3, 7.7] (95)	4.9 [1.1, 7.2] (95) 4.9 [1.4, 9.4] (87)	*<0.001 (C-A)* *0.357 (A-A)*
Plasma EPA (%)					
RCT 1 (0-24wk)	0.8 [0.2, 4.0] (91)	0.8 [0.4, 3.4] (54)	0.8 [0.2, 2.9] (91)	1.4 [0.4, 3.8] (66)]	*<0.001 #*
RCT 2 (0-24wk)	0.8 [0.2, 3.9] (129)	0.7 [0.0, 2.8] (119)	0.8 [0.3, 4.5] (129)	1.7 [0.4, 4.9] (115)	*<0.001*
OLE (24-48wk)			0.7 [0.0, 2.8] (103) 1.7 [0.4, 4.9] (95)	1.9 [0.4, 5.4] (95) 1.7 [0.4, 5.0] (87)	*<0.001 (C-A)* *0.688 (A-A)*
Folate (nM)					
RCT 1 (0-24wk)	15.04 [0.31, 66.70] (71)	16.45 [2.91, 154.8] (63)	14.55 [2.85, 68.46] (76)	44.40 [14.02, 182.0] (66)	*<0.001*
RCT 2 (0-24wk)	12.6 [2.4, 45.3] (129)	13.5 [2.7, 45.3] (119)	12.3 [3.6, 45.3] (128)	37.3 [12.2, 45.3] (115)	*<0.001*
OLE (24-48wk)			13.5 [4.8, 45.3] (103) 37.3 [12.2, 45.3] (95)	37.2 [16.2, 83.4] (95) 39.4 [6.4, 77.1] (87)	*<0.001 (C-A)* *0.405 (A-A)*
Vitamin B12 (pM)					
RCT 1 (0-24wk)	282.0 [71.0, 971.0] (71)	266.5 [68.0, 1265.0] (66)	250.0 [87.0, 676.0] (75)	311.0 [97.0, 856.0] (71)	*<0.001*
RCT 2 (0-24wk)	300.0 [90, 1476] (129)	308.0 [156, 1476] (119)	289.5 [127, 1476] (128)	322.0 [177, 1476] (116)	*<0.001*
OLE (24-48wk)			310.0 [163, 1476] (103) 323.0 [117, 1476] (96)	328.0 [166, 1476] (95) 312.0 [154, 1476] (87)	*0.030 (C-A)* *<0.001 (A-A)*
Vitamin B6 (nM)					
RCT 2 (0-24wk)	45.6 [11.5, 182.3] (45)	42.6 [9.4, 128.0] (41)	37.2 [13.5, 257.2] (37)	59.5 [27.1, 377.4] (36)	*<0.001*

Table 3 *(continued)*

	Control		**Active**		***P-value ****
	Baseline	End of study	Baseline	End of study	
OLE (24-48wk)			43.0 [12.9, 128.0] (38) 60.3 [27.1, 377.4] (33)	84.9 [44.8, 173.8] (17) 76.8 [33.0, 121.9] (13)	*<0.001 (C-A)* *0.244 (A-A)*
Vitamin E (μM)					
RCT 1 (0-24wk)	31.9 [12.3, 66.2] (104)	30.9 [14.3, 78.5] (74)	33.1 [19.2, 75.2] (104)	39.6 [13.1, 83.6] (74)	*<0.001 #*
RCT 2 (0-24wk)	32.0 [9.2, 70.6] (129)	33.2 [14.4, 61.6] (119)	32.1 [18.0, 71.4] (129)	41.6 [25.7, 73.6] (116)	*<0.001*
OLE (24-48wk)			32.8 [14.4, 61.6] (103) 41.7 [25.7, 73.6] (96)	40.2 [20.7, 72.4] (93) 41.5 [20.1, 68.6] (88)	*<0.001 (C-A)* *0.319 (A-A)*
RCT 3 (0-24wk)	29.9 [8.5, 99.8] (255)	30.6 [6.0, 78.4] (233)	29.6 [1.8, 84.3] (260)	38.5 [17.1, 109.4] (239)	*<0.001*
Selenium (μM)					
RCT 1 (0-24wk)	1.1 [0.6-1.8] (73)	1.0 [0.6-1.5] (68)	1.1 [0.6, 2.1] (75)	1.3 [0.7, 2.0] (72)	*<0.001 #*
RCT 2 (0-24wk)	1.1 [0.3, 1.6] (129)	1.1 [0.5, 1.8] (119)	1.1 [0.6, 1.9] (129)	1.4 [0.7, 2.0] (116)	*<0.001*
OLE (24-48wk)			1.1 [0.5, 1.8] (103) 1.4 [0.7, 2.0] (96)	1.3 [0.8, 1.7] (15) 1.3 [1.1, 1.5] (15)	*0.007 (C-A)* *0.017 (A-A)*
Homocysteine (μM)					
RCT 1 (0-24wk)	11.7 [5.0, 83.0] (104)]	12.0 [6.8, 42.1] (74)	12.5 [6.6, 36.7] (104)	9.7 [4.0, 19.1] (74)	*<0.001 #*
RCT 2 (0-24wk)	11.7 [3.9, 28.3] (129)	13.4 [3.3, 38.8] (119)	12.1 [4.4, 37.3] (129)	10.5 [2.3, 20.3] (116)	*<0.001*
OLE (24-48wk)			13.9 [3.3, 38.8] (103) 10.3 [2.3, 20.3] (96)	9.2 [4.7, 17.1] (93) 9.3 [4.3, 19.0] (86)	*<0.001 (C-A)* *<0.001 (A-A)*
RCT 3 (0-24wk)	10.6 [3.9, 100.5] (256)	11.0 [3.5, 102.5] (234)	10.4 [12.8, 50.5] (260)	9.8 [2.0, 46.1] (239)	*0.004*

Data are median [min, max] (n) for all parameters and RCTs to present comparable data. -, not done.

* Mann-Whitney U test, change from baseline at week 24, control vs. active (RCT 1, 2 and 3); Wilcoxon signed-rank test, 24 vs. 48 weeks within control-active (C-A) and active-active (A-A) group (OLE).

Independent samples t-test, change from baseline at week 24, control vs. active.

Table 4 Descriptive statistics for plasma markers of inflammation and oxidative stress; RCT 1.

	Baseline	Week 24	*P-value* *
CRP (mg/L)			
Control	1.75 [0.00, 66.90] (44)	1.80 [0.20, 58.90] (45)	*0.686*
Active	2.00 [0.10, 17.40] (47)	1.80 [0.10, 16.40] (47)	
IL-1β (pg/mL)			
Control	0.24 [0.20, 0.66] (25)	0.24 [0.20, 1.48] (23)	*0.309*
Active	0.24 [0.20, 0.68] (30)	0.24 [0.20, 0.82] (25)	
IL-6 (pg/mL)			
Control	2.67 [0.23, 108.3] (26)	2.59 [0.93, 6.63] (23)	*0.733*
Active	2.65 [0.26, 8.65] (31)	3.20 [0.31, 10.62] (25)	
IL-10 (pg/mL)			
Control	0.91 [0.40, 2.63] (25)	1.01 [0.31, 1.72] (23)	*0.799*
Active	1.06 [0.05, 2.88] (32)	1.00 [0.11, 2.68] (27)	
8-isoprostane (pg/mL)			
Control	17.78 [4.55, 80.85] (25)	22.12 [8.81, 38.40] (23)	*0.071*
Active	19.06 [6.01, 36.32] (30)	17.66 [5.73 - 35.66] (25)	
MDA (μmol/L)			
Control	1.23 (0.53) [45]	1.45 (0.57) [45]	*0.786* #
Active	1.22 (0.56) [47]	1.48 (0.54) [47]	

Data are mean (SD) [n] or median [min, max] (n).

* Mann-Whitney test, change from baseline at Week 24, control vs. active.

Independent samples t-test, change from baseline at Week 24, control vs. active.

Plasma markers of inflammation and oxidative stress

Plasma markers of inflammation (CRP, IL-1β, IL-6 and IL-10) and oxidative stress (8-isoprostane and MDA) were measured at baseline and week 24 in subgroups of the RCT1 study population. No statistically significant between-group differences were observed for the change in any of these parameters (Table 4).

Other micronutrients and fatty acids

Results for plasma Hcy have been published in part before[14-16,19]. They showed significantly decreased levels in the active group versus the control group from baseline to week 24 in RCT1, RCT2 and RCT3 ($p<0.001$ [RCT1 and RCT2] and $p=0.004$ [RCT3], Mann-Whitney *U* test). During the OLE, plasma Hcy levels were significantly decreased from week 24 to Week 48 in the

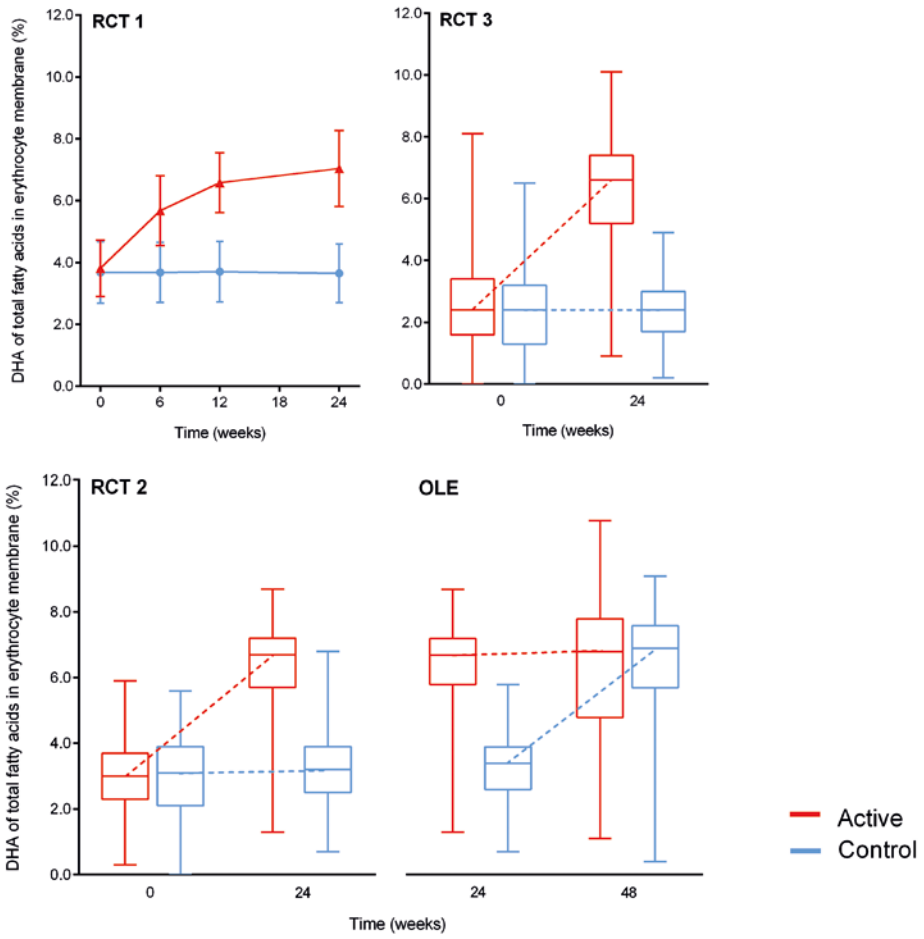

Figure 1

Docosahexaenoic acid (DHA) of total fatty acids in erythrocyte membrane (%) in randomized clinical trial 1 (RCT1), RCT2, RCT3 and the open-label extension (OLE) study in the active and control groups. RCT1, Mean±SD; RCT2,3 and OLE, boxplots show median values (solid horizontal line), 25th-75th percentile values (box outline) and minumum and maximum values (whiskers). Dashed lines connect median values between time points within groups

control-active group ($p<0.001$, Wilcoxon signed-rank test), and continued to decrease within the active-active group ($p<0.001$, Wilcoxon signed-rank test).

The percentage DPA of total fatty acids in the erythrocyte membrane was significantly decreased in the active versus the control group from baseline to week 24 in RCT2 and RCT3 ($p<0.001$,

Mann-Whitney *U* test). During the OLE, levels remained consistently decreased in the active-active group and significantly decreased in the control-active group from week 24 to week 48 ($p<0.001$, Wilcoxon signed-rank test).

For plasma levels of uracil, uric acid, pre-albumin (RCT1), vitamin D and DPA (RCT2), and albumin (RCT1 and RCT2), no statistically significant between-group differences were observed for the change in any of these parameters (data not shown). Cytidine was not detectable in the plasma samples using the current laboratory method.

Discussion

Circulating levels of micronutrients and fatty acids, including uridine, selenium, folate, vitamin B12, vitamin E, vitamin C, DHA and EPA, are reported to be decreased in the AD population and can be increased by 12-48-week oral supplementation with Souvenaid. All micronutrients and fatty acids present in this specific nutrient combination (containing Fortasyn Connect) show increased plasma (and erythrocyte for fatty acids) concentrations after 24 weeks of daily use of active product versus control product in patients with mild and mild to moderate AD, except for vitamin C which could not be reliably measured. Most nutrients remain unchanged during prolonged intake for another 24 weeks. Data derived from RCT1 on vitamin E and percentage DHA and EPA of total fatty acids in erythrocyte membrane suggest that circulating levels are increased already at week 6 and that a plateau is reached within 12 weeks of daily intake (shown for DHA in Figure 1, upper left panel). This is supported by studies showing rising plasma levels within hours of administration of, for example, UMP[11], vitamin C[34], and vitamin E[35]. Together these findings suggest that other micronutrients investigated in this study might follow a similar pattern of increase, as shown for the percentage DHA in the erythrocyte membrane (Figure 1).

All components of Fortasyn Connect are precursors or cofactors required for neuronal membrane formation[36]. Increasing availability of all substrates for the formation of phosphatidylcholine (PC) and phosphatidylethanolamine (PE) is important to enable neuronal membrane synthesis and ultimately support synapse maintenance and/or promote synapse formation[13,37]. Increases in the substrates could underlie the improvement in memory domain scores that were found in the trials in drug-naïve patients with mild AD after 12 weeks (RCT1), 24 weeks (RCT1, RCT2, and OLE) and the exploratory data for the additional 24 weeks (OLE)[14,15,18]. No change in cognition was found, however, after 24 weeks in the clinical trial in patients with mild-to-moderate AD and on AD medication (RCT3)[16]. These results suggest that early intervention is likely key to an effect on cognition.

This is the first study, to the best of our knowledge, in which the effects of sustained dietary intake of UMP on plasma uridine levels in humans are reported. We showed previously that intake of a single serving of 625 mg UMP by healthy human subjects resulted in peak plasma uridine

concentration of 14.6 μmol/L at 1 hour after intake[11]. The present data indicate that daily intake of the same UMP-containing intervention for 24 and 48 weeks increases fasting (RCT1) and non-fasting (RCT2, RCT3, OLE) plasma uridine in people with AD. This new observation is particularly relevant in view of recent studies which indicate that plasma uridine[10,38] and cerebrospinal fluid (CSF) uridine[39,40] levels are lower in AD than in controls. Other investigations indicated that circulating and brain levels of DHA and choline are lower in people with AD than in controls[41-44]. The results of the present study indicate that, in addition to uridine, the investigational product increases circulating DHA and choline, a finding in line with other intervention studies showing that supplemental dietary DHA and choline increase their circulating levels in various populations[45-48]. Uridine together with DHA and choline are the rate-limiting precursors via the Kennedy pathway for the synthesis of phospholipids in neuronal membranes, which are depleted in AD[49,50]. As combined dietary enrichment of these nutrients has been reported to promote the synthesis of brain phospholipids, hippocampal dendritic spines, and synaptic proteins, all prerequisites for synaptogenesis[51], repletion of DHA, choline and uridine may therefore contribute to counteract the characteristic synaptic loss in AD. Tissue target levels of DHA, choline and uridine are not well defined for the general population; however, given their role in neuronal membrane synthesis people with AD likely require higher levels to compensate for membrane loss. The present study indicates that the investigated intervention is efficacious in enhancing circulating levels of these nutrients in AD.

In RCT1, markers of inflammation and oxidative stress were measured as exploratory outcomes to investigate whether the n-3 LC-PUFA and the antioxidants vitamins C and E had a direct effect on inflammation or oxidative stress, respectively. No changes were found, however, in plasma markers of inflammation (CRP, IL-1β, IL-6 and IL-10) or oxidative stress (8-isoprostane and MDA) in a subgroup of the RCT1 population after 24 weeks intake of this specific nutrient combination compared with a control product. Similarly to Freund-Levi et al.[52], who did not find an effect on inflammatory markers in either plasma or CSF, we found no effects on any of the markers of inflammation, even though the current intervention contained two to three times greater amounts of DHA and EPA. In some studies in which researchers did find an effect of antioxidant supplements on markers of oxidative stress in patients with AD contained much higher doses of for example vitamin E (3500 % versus 400% of RDA)[53] than those used in the current study, while others used comparable dosages (200% RDA)[54]. Differences still exist however in the timing and duration of the intervention, and in the method used to measure oxidative stress. These results suggest that changes in cognition found in the trials and the OLE were not due to mediation of inflammation or oxidative stress.

As expected on the basis of equal amounts of vitamin D, protein and energy contained in the active and control product, plasma levels of prealbumin (RCT1), vitamin D (RCT2), and albumin (RCT1 and RCT2) were not altered by intake of this specific nutrient combination.

Plasma Hcy levels were significantly decreased after 24 weeks in the active group and continued to decrease with prolonged intake. This is in line with the observed increases in vitamin B6 and B12, and folic acid levels. Increased B-vitamin levels and decreased Hcy levels enhance methylation capacity, thereby increasing PE to PC conversion by the PE-N-methyltransferase (PEMT) pathway in the liver. This leads to increased availability of choline and DHA-rich PC for (neuronal) membrane synthesis[24,55]. In addition, decreased Hcy levels may indicate improved vascular health. Previous animal studies showed increased cerebral blood flow in a 12 month old mouse model of AD fed this specific nutrient combination compared with a control diet[56].

The increase in percentage EPA of total fatty acids in erythrocyte membrane after 24 weeks in the active group in RCT2 was larger in women than in men. This could be a true sex difference, as Burdge et al.[57,58] showed a higher conversion rate of α-linolenic acid to EPA (and DHA) in young women than in young men, or it could be due to differences in body weight. Flock et al.[59] showed that body weight significantly improved prediction of treatment response of EPA+DHA supplements on erythrocyte membrane content of EPA compared with dose only. However, a trend for an increased treatment response in women compared with men was found in this same study, while already correcting for body weight, suggesting an effect of sex independent from body weight. Despite the uncertainty about the underlying mechanism, the incorporation of EPA in erythrocyte membrane is apparently greater in our population of elderly women with AD than in men, although it was significant in both sex groups.

DPA erythrocyte (but not plasma, RCT2) concentrations were decreased from baseline to week 24 in the active group compared with the control group in RCT2 and 3, and reached a plateau in the OLE at week 48. Possibly the increasing availability of the two other major n-3 LC-PUFAs, DHA and EPA, reduces the incorporation of DPA in erythrocytes while plasma levels remain stable[60].

The data presented in this article on the increased levels of multiple nutrients and fatty acids are generally in line with known kinetics of single nutrients, which assures that there is no relevant interaction in absorption. We found consistent results over three large RCTs when we evaluated the ITT population, representing patients with both mild and mild–to-moderate AD, and including patients using AD medication as well as drug-naïve patients. However, we report on exploratory analyses and none of our outcome measures were primary endpoints of the studies. Furthermore, it should be noted that the present data refer only to circulating levels, whereas the nutrients should have their effect in the brains of patients with AD. With the current data we cannot confirm whether these nutrients or their metabolites cross the blood-brain barrier and have an effect on synapse synthesis and maintenance. Evidence exists, however, to support the proposed mechanism of action. On the basis of tracing studies done with positron emission topography (PET), we know that at least the precursors for the Kennedy pathway, i.e. choline, DHA and uridine do reach the brain within hours of administration in animals and/or healthy

humans[61-63]. For instance, Umhau et al.[62] showed that, based on PET measurements 60 minutes following administration of [1-^{11}C]DHA, the incorporation rate of DHA could be calculated in healthy human volunteers. Additionally, in a subset of the study population of RCT2 additional electroencephalographic measurements were performed to assess underlying synaptic function. We found preserved organization of brain networks in patients with mild AD within 24 weeks, compared with a control product, hypothetically counteracting the progressive network disruption over time in AD[64]. Currently, a study of drug-naïve patients with mild AD is ongoing to investigate the effect of this specific nutrient combination on brain phospholipid metabolism by phosphorus magnetic resonance spectroscopic imaging (MRSI)[65]. In contrast to the present study, this MRSI study will provide more direct evidence on the extent to which the nutrients and their metabolites can affect neuronal membrane turnover and influence synaptic function.

Conclusion

These data show that circulating levels of nutrients, known to be decreased in the AD population, can be increased in patients with mild and mild-to-moderate AD by 24-48 weeks oral supplementation with Souvenaid. Uptake is observed within 6 weeks and a plateau phase is reached for most nutrients during prolonged intake, thus increasing the availability of precursors and cofactors necessary for the formation and function of neuronal membranes and synapses for the brain. This adds to the rationale of using oral supplementation of micronutrients to replenish nutritional deficits in patients with AD.

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APPENDIX A

List of Independent Ethics Committees or Institutional Review Boards

Souvenir I

The Netherlands:
- VU medical centre, Amsterdam
- Radboud University medical centre, Nijmegen
- Academic Hospital Maastricht, Maastricht
- Jeroen Bosch Hospital, 's-Hertogenbosch
- University Medical Centre Utrecht, Utrecht
- Medical Centre Alkmaar, Alkmaar
- University Medical Centre Groningen, Groningen
- Gelderse Vallei Hospital, Ede
- Tergooiziekenhuizen, Blaricum
- Diaconessenhuis Leiden, Leiden
- Amphia Hospital, Breda

Belgium:
- University Hospital Gasthuisberg, Leuven
- AZN Middelheim, Antwerpen
- Virga Jess kliniek, Hasselt
- Universitair Ziekenhuis Gent, Gent
- Heilig Hart ziekenhuis, Roeselare
- Sint Andries ziekenhuis, Tielt

Germany:
- Ethikkommission der Fakultät für Medizin der Technischen Universität München, München
- Ethikkommission der Sächsische Landesärztekammer, Dresden
- Ethikkommission Medizinische Fakultät Carl Gustav Carus der Technische Universität Dresden, Dresden

Germany (continued):
- Ethikkommission der Medizinischen Fakultät Heidelberg, Heidelberg
- Ethik-Kommission der Ärtzekammer Hamburg, Hamburg
- Ethik-Kommission der Georg-August Universität Göttingen, Göttingen
- Ethik-Kommission der Medizinischen Fakultät der Ruhr-Universität Bochum, Bochum
- Ethikkommission der Ärztekammer Nordrhein, Düsseldorf
- Ethik-Kommission der Ärztekammer Schleswig-Holstein, Bad Segeberg
- Ethik-Kommission bei der Landesärtztekammer Hessen, Frankfurt am Main
- Ethik-Kommission der Bayerischen Landesärtztekammer, München
- Ethik-Kommission der Georg-August Universität Göttingen, Göttingen
- Ethik-Kommission der Medizinischen Fakultäat der Ruhr-Universität Bochum, Bochum
- Ethik-Kommission der Ärztekammer Nordrhein, Düsseldorf

United Kingdom:
- Southampton & South West Hampshire REC, Southampton
- Bath Local Research Ethics Committee
- Swindon Research Ethics Committee
- Bradford Research Ethics Committee
- Cumbria and Lancashire Ethics Committee

United States:
- Institutional Review Board Saint Louis University Care, St. Louis

Souvenir II

The Netherlands

For central approval

- Medisch Ethische Toetsingscommissie VUmc, Amsterdam

For assessment of local feasibility

- Medisch Ethische Commissie azM/UM, Maastricht
- Commissie Mensgebonden Onderzoek regio Arnhem-Nijmegen
- Medisch Ethische Toetsingscommissie Catharina Ziekenhuis, Eindhoven
- Medisch Ethische Toetsingscommissie Amphia, Breda
- Wetenschapsbureau Jeroen Bosch Ziekenhuis, Den Bosch
- LAWO Orbis Medisch Centrum (Lokale Adviesgroep Wetenschappelijk Onderzoek), Sittard
- Regionale Toetsingscommissie Patiëntgebonden Onderzoek, Leeuwarden
- Medisch Ethische Toetsingscommissie Tergooiziekenhuizen, Blaricum

Belgium

For central approval

- Commissie Medische Ethiek ZNA/ O.C.M.W., Antwerp

For local positive opinion

- Comité voor Medische Ethiek, Sint-Andriesziekenhuis Tielt
- Medische Ethische Commissie H. Hartziekenhuis, Roeselare
- Ethisch Comité vzw Emmaüs, AZ Sint-Maarten, Mechelen
- Ethisch Comité Sint-Trudoziekenhuis, Sint-Truiden

Germany

For central approval by each EC

- Ethik-Kommission der Universität Ulm
- Ethik-Kommission der Ärztekammer Nordrhein
- Ethik-Kommission der sächsischen Landesärztekammer
- Ethik-Kommission der Ärztekammer Westfalen-Lippe und der Medizinischen Fakultät der Westfälischen Wilhelms-Universität Münster
- Ethikkommission der medizinischen Fakultät Heidelberg
- Ethik-Kommission der Bayerischen Landesärztekammer
- Ethik-Kommission der Medizinischen Fakultät der Universität zu Köln Forum

Spain

For central approval by each EC

- Comité ético de investigación clínica. Hospital de la Santa Creu i Sant Pau, Barcelona
- Comité ético de investigación clínica. Hospital Clínico San Carlos, Madrid
- H.Clinic I Provincial EC Agencia de Ensayos Clínicos, Hospital Clinic de Barcelona.
- Hospital Virgen Arrixaca EC, Murcia

Italy

For central approval by each EC

- Comitato Etico Fondazione Ospedale Maggiore, Milan
- Comitato Etico Aziende Sanitarie Umbria
- Comitato Etico dell'Università Cattolica del Sacro Cuore, Rome
- Comitato Etico dellâ´Azienda Ospedaliera Universitaria s. Martino di Genova

France

For central approval

- CPP sud-ouest, Toulouse

Open-label extension

The Netherlands

For central approval

- Independent Review Board Nijmegen (IRBN), Nijmegen

For assessment of local feasibility

- Medisch Ethische Toetsingscommissie VUmc, Amsterdam
- Medisch Ethische Commissie azM/UM, Maastricht
- Commissie Mensgebonden Onderzoek regio Arnhem-Nijmegen
- Medisch Ethische Toetsingscommissie Catharina Ziekenhuis, Eindhoven
- Medisch Ethische Toetsingscommissie Amphia, Breda
- Wetenschapsbureau Jeroen Bosch Ziekenhuis, Den Bosch
- LAWO Orbis Medisch Centrum (Lokale Adviesgroep Wetenschappelijk Onderzoek), Sittard
- Regionale Toetsingscommissie Patiëntgebonden Onderzoek, Leeuwarden
- Medisch Ethische Toetsingscommissie Tergooiziekenhuizen, Blaricum

Belgium

For central approval

- Commissie Medische Ethiek ZNA/ O.C.M.W., Antwerp

For local positive opinion

- Comité voor Medische Ethiek, Sint-Andriesziekenhuis Tielt
- Medische Ethische Commissie H. Hartziekenhuis, Roeselare
- Ethisch Comité vzw Emmaüs, AZ Sint-Maarten, Mechelen
- Ethisch Comité Sint-Trudoziekenhuis, Sint-Truiden

Germany

For central approval by each EC

- Ethik-Kommission der Universität Ulm
- Ethik-Kommission der Ärztekammer Nordrhein
- Ethik-Kommission der sächsischen Landesärztekammer
- Ethikkommission der medizinischen Fakultät Heidelberg
- Ethik-Kommission der Bayerischen Landesärztekammer

Spain

For central approval by each EC

- Comité ético de investigación clínica. Hospital de la Santa Creu i Sant Pau, Barcelona
- Comité ético de investigación clínica. Hospital Clínico San Carlos, Madrid
- H.Clinic I Provincial EC Agencia de Ensayos Clínicos, Hospital Clinic de Barcelona.

Italy

For central approval by each EC

- Comitato Etico Fondazione Ospedale Maggiore, Milan
- Comitato Etico Aziende Sanitarie Umbria
- Comitato Etico dellâ´Azienda Ospedaliera Universitaria s. Martino di Genova

France

Central approval

- CPP sud-ouest, Toulouse

S-Connect

United States

Quorum Review IRB as the central Ethics Committee for sites 013, 040, 049, 022, 005, 034, 028, 047, 056, 025, 045, 051, 052, 041, 053, 004, 042, 024, 050, 021, 017, 039, 046, 029, 002, 057, 023,032, 036, 037, 043, 048, 020, 027, 033 (see also chapter 2 for detailed site information).

For site 011: Medical College of Wisconsin, IRB Human Research Review Committee

For site 014: University of Kansas Medical Center, Human Subjects Committee

For site 003: Indiana University, IRB

For site 008: University of Pennsylvania, IRB

For site 035: University of Kentucky, Medical IRB

For site 044: Drexel University College of Medicine, IRB

For site 038: Medical University of South Carolina, IRB

For site 006: Saint Louis University, IRB (Biomedical)

For site 011: Oregon Health and Science University, IRB

For site 012: University of Florida, IRB

For site 007: University of Texas Health Science Center, IRB

For site 001: Rush University Medical Center, IRB

For site 016: Wake Forest University Health Sciences, IRB

Study centres S-Connect

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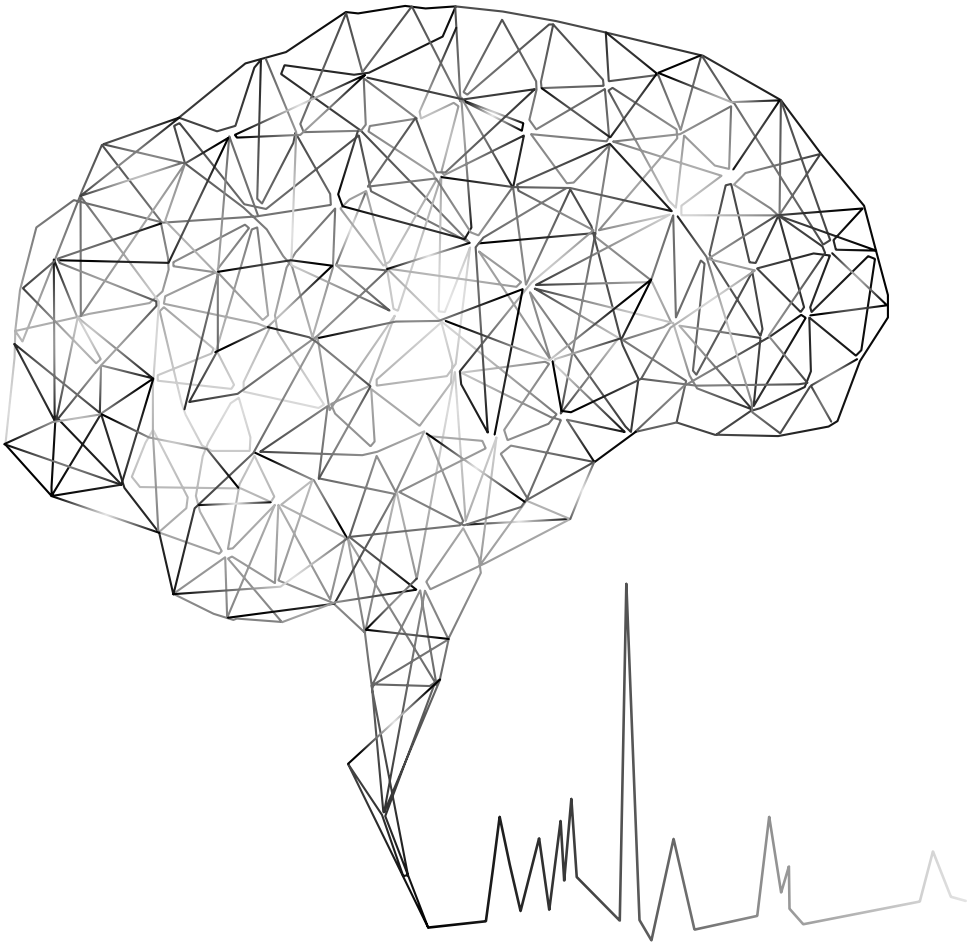
3

Altered brain high-energy phosphate metabolism in mild Alzheimer's disease



Anne Rijpma, Marinette van der Graaf, Olga Meulenbroek, Marcel G.M. Olde Rikkert, Arend Heerschap. Altered brain high-energy phosphate metabolism in mild Alzheimer's disease.

Submitted.

Abstract

Defects in essential metabolic processes for energy supply and phospholipid membrane function have been implicated in Alzheimer's disease (AD). Post-mortem investigations are generally limited to late stage disease and prone to tissue decay artifacts. *In vivo* assessments of high energy phosphates, tissue pH and phospholipid metabolites are possible by phosphorus MR spectroscopy (^{31}P-MRS), but so far only small studies, mostly focusing on single brain regions, have been performed. Therefore, we assessed phospholipid and energy metabolism in multiple brain regions of 31 early stage AD patients and 31 age- and gender-matched controls using ^{31}P-MRS. An increase of phosphocreatine (PCr) was found in AD patients compared with controls in the retrosplenial cortex, and both hippocampi, but not in the anterior cingulate cortex. While PCr/inorganic phosphate and pH were also increased in AD, no changes were found for phospholipid metabolites. This study showed that PCr levels are specifically increased in regions that show early degeneration in AD. Together with an increased pH, this indicates an altered energy metabolism in mild AD.

Introduction

Alzheimer's disease (AD) is the main cause of dementia in the elderly, responsible for about half of the nearly 47 million dementia cases worldwide[1]. Although the disease is defined by the accumulation of amyloid beta plaques and neurofibrillary tau tangles in the brain[2], other pathological processes can be identified such as oxidative stress, vascular dysfunction, and inflammation[3-5]. Additionally, defects in essential metabolic processes for energy supply and membrane function have been implicated in AD[6,7].

The human brain is highly vulnerable to disturbances in energy metabolism, due to its relatively large energy consumption. Previous AD studies demonstrated alterations in global and cellular energy metabolism. FDG-PET studies have shown glucose hypometabolism in the retrosplenial cortex (RSC) and medial temporal lobe in people with mild cognitive impairment (MCI) and AD patients, as well as in cognitively normal carriers of the APOE ε4 allele[8-11]. Furthermore, the enzyme creatine kinase (CK), obtained from post-mortem AD tissue, shows reduced activity compared with samples that are free from neurological disease[12,13]. The CK reaction, key to balance brain energy metabolism, can quickly replenish ATP from the energy buffer phosphocreatine (PCr), when local energy demands suddenly increase[14]. This reaction also enhances the efficiency of mitochondrial oxidative phosphorylation (OXPHOS) by keeping adenosine diphosphate (ADP) sufficiently available [15] and prevents acidification by maintaining pH (for a review[16]).

One of the earliest pathological changes in AD is synaptic dysfunction, which correlates well with cognitive dysfunction and disease severity[17]. Alterations in neuronal membrane composition, vital for synaptic transmission, have been linked to synaptic dysfunction in AD[18]. For instance, post mortem studies found reduced levels of the major membrane components phosphatidylcholine, phosphatidylethanolamine and phosphatidylinositol[7,19], as well as altered activity of catabolic and anabolic enzymes, suggesting compensatory metabolic changes to reduce the rate of neuronal membrane loss[20].

High energy phosphates, such as ATP and PCr, and metabolites of phospholipid membrane metabolism can be assessed *in vivo* by phosphorus magnetic resonance spectroscopy (^{31}P-MRS). This technique has been applied in AD e.g. [21,22-24], but previous studies, essentially focusing on single brain regions, were severely hampered by small sample sizes and low spectral resolution, resulting in a wide disparity in findings. Furthermore, differences in the control groups that were used, in the brain regions that were studied and in the disease stages and medication statuses of the patients, may have contributed to apparent inconsistencies in the literature.

In the current study we tried to overcome these limitations, supported by recent technical advances. First, MRS imaging (MRSI) allows us to investigate multiple brain regions simultaneously. We specifically addressed regions that are of interest in AD, namely, the hippocampus, the RSC, and

the anterior cingulate cortex (ACC). Secondly, we utilized proton decoupling at 3T field strength, to increase spectral resolution and sensitivity. Finally, we selected a well-defined patient group and an age- and gender-matched control group. Accordingly, the purpose of our study was to assess phospholipid and energy metabolism in multiple brain regions in mild AD patients and healthy age-matched control subjects using 3D ^{31}P MRSI.

Materials and Methods

Subjects and design

All visits of patients and controls to the Radboud university medical center (Nijmegen, the Netherlands) took place between 2012 and 2015. Patients aged ≥ 50 years with a diagnosis of possible or probable AD according to the revised National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA) 2011 criteria[25] with evidence of the pathophysiological process (i.e. from structural MRI or cerebrospinal fluid biomarker assays) and with a minimum Mini Mental State Examination (MMSE) score of 20 were recruited from the hospital's memory clinic or by referral from regional hospitals. All AD patients participated in a randomized controlled trial on the effect of a medical food on brain phospholipid metabolism (Dutch Trial Register 3346). Only baseline data were used in the current study. Healthy control subjects, age- and gender-matched to the AD group, were screened by telephone before being invited to the clinic. All subjects were drug-naïve for AD medication (cholinesterase inhibitors and NMDA-antagonists) and were free from neurological or psychiatric disorders (other than dementia, for the AD patients). Patients nor healthy controls used nutritional supplements containing docosahexaenoic acid, eicosapentaenoic acid or > 200% of the recommended daily allowance of vitamins B, C, or E. All subjects were screened for MRI contra-indications before inclusion in the study.

Written informed consent was obtained from all subjects and from the informal caregivers of the AD patients. The local ethics committee reviewed and approved the protocol and the study was conducted in accordance with the Declaration of Helsinki.

Medical history, medication use and MMSE were recorded from all subjects, as well as date of birth, sex, ethnicity, smoking habits, alcohol consumption, and family history of AD. Level of education was classified according to Verhage[26] (comparable with the International Standard Classification of Education) and categorized in three groups: primary education or lower (low), junior vocational training (middle), and senior vocational or academic training (high).

MR acquisition

MRI and MRS were performed on a Magnetom Tim Trio 3T MR system (Siemens, Erlangen, Germany) with a dual-tuned ^{1}H/^{31}P volume head coil (Rapid Biomedical, Wuerzburg, Germany).

High resolution MR images were acquired with a T1-weighted magnetisation-prepared rapid gradient-echo sequence (TR=2300 ms, TE=3.16 ms, 15° flip-angle, 176 sagittal slices, slice-matrix size=256×256 mm, slice thickness=1 mm, voxel-size=1×1×1 mm, TA=6:25 min).

^{31}P-MR spectra were acquired with whole brain 3D MRSI (TR=2000 ms, acquisition delay=0.10 ms, 40° flip-angle, WALTZ4 proton decoupling at 50% of 512 ms acquisition duration, FOV 260x260x260 mm; matrix size=10x10x10, acquisition time 13:08 min). K-space was sampled with a weighted elliptical phase encoding scheme with four averages. The volume of interest was centered on the midline and parallel to the line from the anterior commissure to the posterior commissure. Spatial post-processing consisted of zerofilling to a matrix size of 16x16x16. The nominal volume of the measured voxels is about 17.5 cc, the effective voxel at 64% of the spatial response function has a spherical shape with a volume of about 40 cc[27].

MRS and MRI data analyses

Four regions of interest (ROI) were selected for analysis from the 3D ^{31}P-MRSI data: ACC, RSC, and left and right hippocampus (HL and HR). Guided by the T1-weighted images, the MRSI grid was shifted in the x, y and z dimension to position voxels in the ROI's (Figure 1). For the anterior cingulate cortex (ACC), a voxel was selected anterior to the genu of the corpus callosum, with the inferior border aligned with the the line from the anterior commissure to the posterior commissure (AC-PC line), and on the midsagittal plane. For the retrosplenial cortex (RSC), a voxel on the midsagittal plane was selected posterior to the splenium of the corpus callosum, with the inferior border aligned with the AC-PC line. For left and right hippocampus (HL and HR), a voxel was selected at the anterior end of each hippocampus, with the inferior and lateral border of the voxel aligned with the inferior and lateral side of the hippocampus.

The software package Metabolite Report (Siemens Healthcare, Erlangen, Germany) was used for post-processing (i.e. 100 ms exponential filter, zero-filling, phase correction, baseline correction) and for automatic fitting of the spectra in the time-domain using prior knowledge appropriate for ^{31}P MR spectra (own spectral data and de Graaf[28]). Eleven well-resolved resonance peaks were fitted: the phospholipid metabolites phosphoethanolamine (PEth), phosphocholine (PCh), glycerophosphoethanolamine (GPEth) and glycerophosphocholine (GPCh), the high-energy phosphorus molecules PCr, ATP (α-ATP, β-ATP and γ-ATP), nicotinamide adenine dinucleotide (NAD(H)) and inorganic phosphate (Pi), and membrane-bound phospholipid. In this procedure, PCh (6.7 ppm; bounds 6.6-7.5 ppm), PEth (6.3 ppm; bounds 6-6.55 ppm), GPCh (3 ppm; bounds 2.8-3.2 ppm), GPEth (3.6 ppm; bounds 3.2-3.8 ppm), PCr (0 ppm; bounds -0.5-0.5 ppm), and inorganic phosphate (Pi) (4.8 ppm; bounds 4.5-5.7 ppm) were each modeled as a single Gaussian peak (default line width of 15 Hz, variable between 1 and 60 Hz). PCh, PEth and Pi were constrained to the same line width, and GPCh and GPEth were constrained to the same line width. Membrane phospholipids (MP) (2.25 ppm, bounds 2-2.4 ppm) were fitted with a single Gaussian peak, with a line width of 65 Hz (variable between 30 and 80 Hz). The γ and

α resonances of adenosine-5'-triphosphate (ATP) were each fitted as a doublet with two single Gaussian peaks with a constrained separation according to their J-coupling constants (γATP -2.5 ppm, -0.28; bounds -3--2 ppm; αATP -7.5 ppm, -0.327; bounds -9--7 ppm) [28], and forced equal amplitude. The single peaks were constrained to the same line widths (default 15 Hz, variable between 5 and 80 Hz). The β resonance of ATP was fitted as a triplet with three single Gaussian peaks with a constrained separation according to the J-coupling constant (-16.3 ppm +/- 0.325; bounds -18--14 ppm)[28]. The two outer peaks were forced to an amplitude equal to half the intensity of the middle peak. Line width was constrained to the line width of the other ATPs. NAD(H) (-8.3 ppm; bounds -9.5--7.5 ppm) was modeled as a single Gaussian peak with line width constrained to the line width of ATP.

Both a quantitative evaluation of the fitting results and a visual quality control were performed. Quantitatively, only metabolite fits with a Cramer–Rao lower bound of ≤30% were considered reliable. Qualitatively, two spectroscopists independently checked the spectra by visual inspection of the original spectra and the fitting results. If a metabolite peak was visually present, and its fit was assigned to the correct resonance—giving a minimal residue in the subtraction spectrum—the fitting result was accepted.

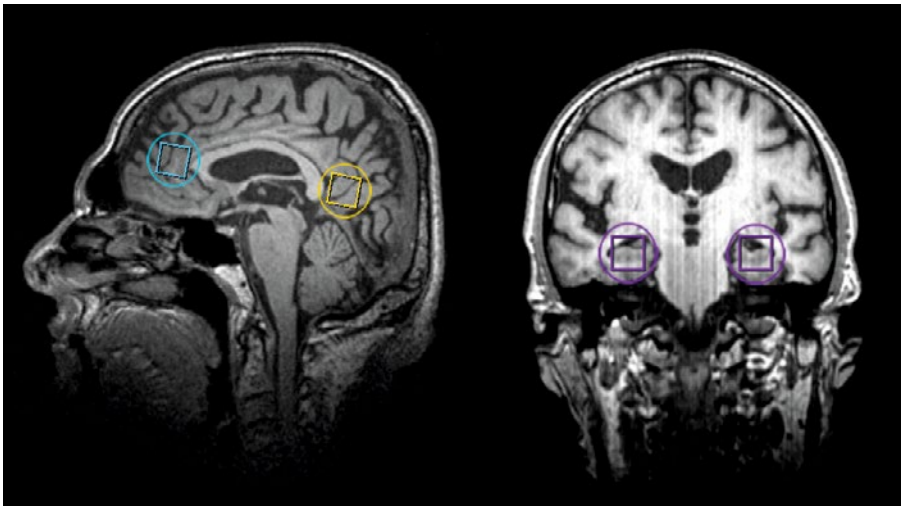


Figure 1

Voxel selection of ^{31}P MR spectra displayed on sagittal (left) and coronal (right) anatomical images: anterior cingulate cortex (blue), retrosplenial cortex (yellow), left and right hippocampus (purple). The nominal voxel size is indicated by squares and the approximation of the effective spherical voxel size is indicated by circles.

The integral of each metabolite resonance was expressed as a percent area of the total phosphorous signal. Hereby, the data were corrected for CSF content and thus for differences in atrophy. Phosphomonoesters (PME) were calculated from the sum of PEth and PCh, phosphodiesters (PDE) from GPEth and GPCh and total ATP (tATP) from the sum of α-ATP, β-ATP and γ-ATP. In addition, the ratios PME/PDE, PE/GPE, PC/GPC and PCr/Pi were computed. Intracellular pH was determined from the chemical shift difference between the PCr and Pi resonance peaks[29].

The T_1-weighted images were segmented into grey matter (GM), white matter (WM) and CSF using automatic segmentation software (SPM8, Welcome Trust Centre for Neuroimaging, London, UK; VBM8, Structural Brain Mapping Group, Jena, USA). Binary tissue maps (matrices) of GM, WM, CSF were convolved with the, digitally sampled, mathematically-modelled three dimensional point-spread function of the MRSI, using Matlab 2012b (The Mathworks, Inc., Matrick, MA), to obtain theoretically correct values of the contribution of tissue type to each ROI (each consisting of one MRS voxel). GM-to-WM ratio (GM/WM) was calculated for each ROI for each subject.

Statistical analyses

Two-factorial mixed ANOVAs were used to test differences in tissue content (GM, WM, and CSF) and GM/WM in each ROI between mild AD patients and controls. Group and brain region (ROI) differences were analyzed for each spectroscopy outcome parameter using a mixed model with group (AD or control), sex, brain region and its interaction with group as fixed factors, considering brain region as a within subject factor, and adjusting for age. An unstructured variance-covariance matrix for brain region was selected. The group-by-brain region interaction was only kept in the model if $p<0.10$. In the covariate analysis, GM/WM in each ROI was added to the final primary model of each parameter, to investigate the effect of tissue composition on the effects of group and brain region. Although the inclusion of GM/WM in the final model led to a small change (10-20 %) in many parameter estimates, this was mainly without a change in main and/or interaction effects. Therefore we report the primary model in tables and figures and refer to the effect of GM/WM only where it led to a change in the main and/or interaction effects, and with > 20% change in the parameter estimates of group or brain region. Goodness of fit was assessed by comparing Bayesian Information Criteria (BIC) and homoscedasticity and normality of residuals were assessed by visual inspection. Statistical analyses were performed using SAS 9.2 Software for Windows (SAS Institute Inc., Cary, NC, USAf). Statistical significance was set at $p < 0.05$ without correction for multiple testing.

Results

Subjects

A total of 33 mild AD patients and 35 healthy control subjects were enrolled in the study and subjected to MRI and MRS measurements. Two AD patients had MR spectra of low quality (due to severe motion and a dental implant) and four control subjects were found to meet an exclusion criterion during or after the visit (two subjects took nutritional supplements, two subjects had incidental findings indicating neurological disease). Therefore, 31 AD patients and 31 controls were included in the final analysis. The groups were similar with respect to age and gender (see Table 1 for subject characteristics).

Table 1 Subject characteristics

	Alzheimer's disease n=31	**Healthy controls n=31**
Age	73.4 years (6.8)	73.5 years (6.3)
Gender (%)	13 males (42%)	15 males (48%)
MMSE	23.2 (2.0)	28.1 (1.4)
Time since AD diagnosis	1.6 months [0.2-15.3]	-
BMI	25.4 kg/m^2 (3.8)	26.3 kg/m^2 (4.5)
Educational level (low/middle/high)	9/16/6	4/9/18
Smoking (never/past/current)	13/17/1	6/22/3
≥ 1 relatives with AD (n)	19	7

MMSE, mini mental state examination; AD, Alzheimer's disease; BMI, body mass index. Data are presented as mean (standard deviation) or median [range] unless otherwise indicated.

ROI tissue contribution

The interaction between group and brain region for GM and CSF contributions to the total volume of each ROI was not significant, but GM and CSF differed significantly between groups, and between brain regions (all $p<0.001$). GM was lower and CSF was higher in AD patients than in healthy control subjects (mean % ± SD, GM:36.3±4.7 vs. 40.5±4.0; CSF: 22.5±4.7 vs. 18.1±3.5). overall, GM was highest in the right hippocampus, followed by left hippocampus, and then followed by equal amounts in RSC and ACC (data not shown). CSF in RSC and ACC was higher than in both hippocampi. There was a significant interaction between group and brain region for WM ($p=0.004$) and GM/WM ($p=0.021$). WM was higher in AD patients compared with controls in the right hippocampus (41.5±3.0 vs. 40.0±2.6), but not in the other brain regions. GM/WM was lower in AD patients than in controls in ACC, and both hippocampi, but not in RSC (data not shown).

MRS results

High quality spectra were obtained with well-resolved phosphomonoester and phosphodiester signals (Figure 2). We did not observe a difference in the linewidth of PCr between AD and controls (mean±SD 11.5±3.9 Hz and 11.1±3.0 Hz, not tested). ROI tissue contributions showed typical differences with lower GM and higher CSF in AD patients compared with controls.

In the statistical analysis, the interaction between group and brain region was kept in the model for PCr, γATP and βATP, because it survived our criterion of $p<0.10$. For all other outcome parameters, the interaction term had a p-value >0.10 and was removed from the model.

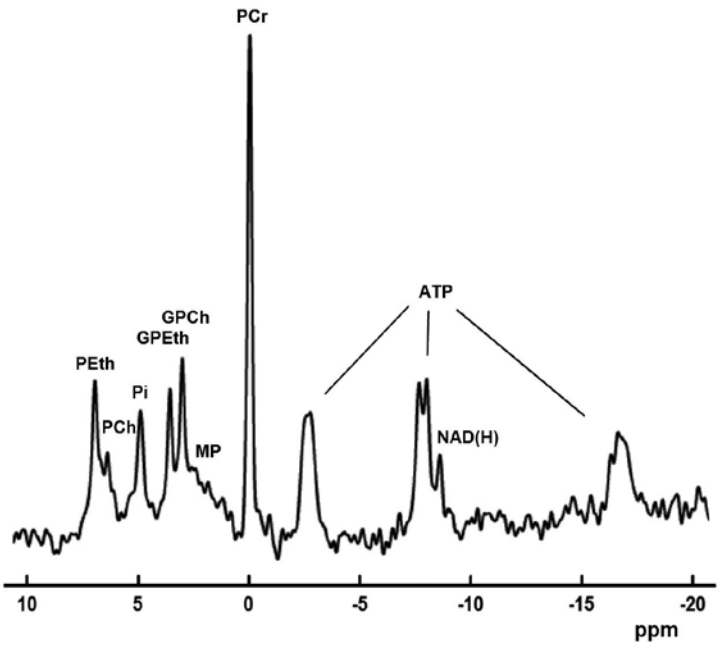


Figure 2

Representative spectrum from the retrosplenial cortex of an Alzheimer's disease patient (73 years old). Zero filling to 4096 data points and an 8Hz Gaussian filter were applied. PEth, phosphoethanolamine; PCh, phosphocholine; Pi, inorganic phosphate; GPEth, glycerophosphoethanolamine; GPCh, glycerophosphocholine; MP, membrane phospholipids; PCr, Phosphocreatine; ATP, adenosine triphosphate; NAD(H), nicotinamide adenine dinucleotide; ppm, parts per million

Differences in energy metabolites and pH between groups

A significant interaction between group and brain region was found for PCr, showing that normalized PCr signals were higher in mild AD patients compared with healthy control subjects

in the RSC (Least Square (LS) mean difference=0.86, SEM=0.29, p=0.004), HL (LS mean difference=1.52, SEM=0.52, p=0.005) and HR (LS mean difference=1.47, SEM=0.38, p<0.001), but not in the ACC (p=0.947) (Figure 3A). The interaction between group and brain region was not significant for γATP and βATP. A main effect of group was found for PCr/Pi, showing that this ratio was higher in mild AD patients compared with healthy controls (LS mean difference=0.22, SEM=0.11, p=0.046) (Figure 3B). Also pH was increased in AD patients compared with controls (LS mean difference=0.008, SEM=0.004, p=0.032) (Figure 3C). No group differences were found for γATP, βATP, αATP, total ATP, Pi, and NAD(H) (all p>0.05). For a summary of group effects see Table 2.

Differences in phospholipid metabolites between groups

No interaction or group difference between mild AD patients and healthy control subjects were found for any of the phospholipid metabolites (PEth, PCh, GPEtn, GPCh, PME, and PDE; Table 2) or their ratios (PEth/GPEth, PCh/GPCh, and PME/PDE) (all p>0.05).

Differences in phosphorus metabolites and pH between brain regions

Brain region significantly affected all outcome parameters (all p<0.05), except PCh and PME (p>0.05). The normalized signals of total ATP, αATP, βATP, γATP, NAD(H), PEth, GPEtn, GPCh, and PDE were lower in the RSC than in the ACC and both hippocampi (all p<0.05), while the pattern was reversed for the ratios PCh/GPCh and PME/PDE (p<0.001). We found no difference between the left and right hippocampus for any of the energy and phospholipid metabolites or pH (p>0.05), except for αATP (LS mean difference=0.65, SEM=0.26, p=0.015). See Table 3 for an overview of the differences between brain regions.

Influence of tissue content

In the covariate analysis GM/WM was added to the final model. This did not change the effects of group, brain region or the interaction between group and brain region on PCr, Pi, total ATP, αATP, βATP, γATP, NAD(H), pH or any of the phospholipid metabolite signals. However, for PCr/Pi the main effect of brain region was reduced to a trend (p=0.062) and the parameter estimates changed substantially, when GM/WM was included in the model. There was no change in the effect of group on PCr/Pi.

Discussion

In this study we obtained high-quality ^{31}P-MRS data in a large group of mild AD patients and in a well-matched healthy elderly population. High spectral resolution enabled us to gather information on high-energy phosphates, as well as on specific phospholipid metabolites. Measurements were performed in multiple brain regions that are among the most investigated in AD and for the first time, these areas were investigated simultaneously by ^{31}P-MRS. We detected

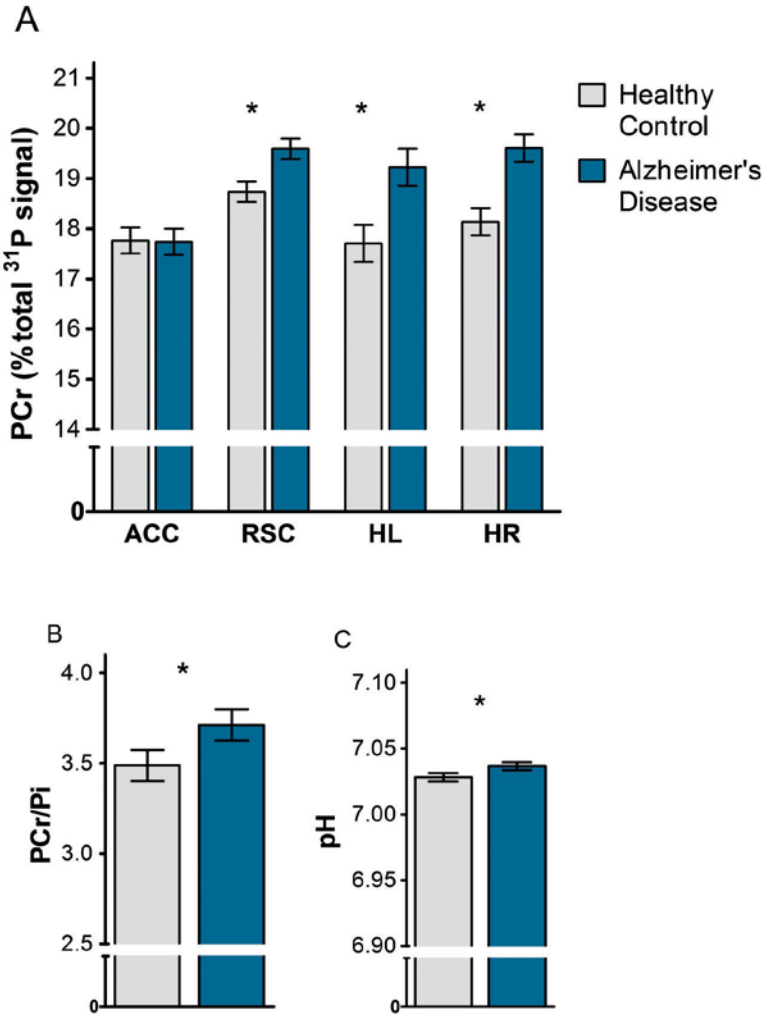


Figure 3

Normalized phosphocreatine (PCr; A), ratio of PCr to inorganic phosphate (PCr/Pi; B) and pH (C) in patients with Alzheimer's disease (AD) and healthy control (HC) subjects. Data represent Least Square means±SEM, *$p<0.05$ AD vs HC. ACC, anterior cingulate cortex; RSC, retrosplenial cortex; HL, left hippocampus; HR, right hippocampus.

Table 2 Main effect of group (healthy controls vs Alzheimer's disease) on phosphorus metabolite signals and tissue pH for all investigated regions

	AD	**Control**	**p-value**
PCr*	19.04±0.18 *n=124*	18.08±0.18 *n=124*	**<0.001**
Pi	5.30±0.11 *n=121*	5.47±0.11 *n=123*	0.240
PCr/Pi	3.71±0.09 *n=121*	3.49±0.09 *n=123*	**0.046**
total ATP	42.88±0.27 *n=124*	43.17±0.27 *n=124*	0.445
NAD(H)	3.96±0.11 *n=103*	4.09±0.11 *n=96*	0.388
PEth	8.19±0.11 *n=119*	8.33±0.11 *n=122*	0.375
PCh	3.90±0.07 *n=112*	3.93±0.07 *n=114*	0.757
GPEth	4.32±0.11 *n=109*	4.31±0.11 *n=117*	0.942
GPCh	5.96±0.15 *n=108*	6.08±0.15 *n=109*	0.526
pH	7.037±0.003 *n=121*	7.028±0.003 *n=123*	**0.032**

LS means ± SEM of metabolite levels, as percentage of total phosphorus signal, in patients with Alzheimer's disease and healthy control subjects. Significant group effects ($p<0.05$, AD versus Control) indicated in bold. *PCr also has a significant interaction between group and brain region. LS, least squares; SEM, standard error of the mean; n, number of observations; AD, Alzheimer's disease; HC, healthy control; PCr, Phosphocreatine; Pi, inorganic phosphate; ATP, adenosine triphosphate; NAD(H), nicotinamide adenine dinucleotide; PEth, phosphoethanolamine; PCh, phosphocholine; GPEth, glycerophosphoethanolamine; GPCh, glycerophosphocholine

increased phosphocreatine and pH, but no changes in phospholipid metabolite signals in mild AD patients. Interestingly, the increase in PCr was observed in the RSC and in the hippocampi, regions that both show early pathological changes in AD, but not in the ACC, a region known to be involved in the disease at a later stage. This suggests that the PCr abnormality expands across brain regions in a similar fashion as other AD pathologies, such as tau[2]. Furthermore, in both groups pH, high energy phosphates, and most phospholipid metabolites differed across brain regions.

As *in vivo* human ^{31}P MRS brain studies are nearly always performed under signal saturating conditions, changes in T1 relaxation time may affect metabolite signal intensities[30]. Although we cannot exclude that such an effect has contributed to the increased PCr signal, it requires

Table 3 Main effect of brain region on phosphorus signal integrals and tissue pH

	ACC	RSC	HL	HR	p-value
PCr*	17.75±0.18†,‡,§ *n=62*	19.16±0.14¶ *n=62*	18.47±0.26 *n=62*	18.87±0.19 *n=62*	**<.001**
Pi	5.34±0.12 *n=61*	5.65±0.10¶ *n=62*	5.21±0.16 *n=61*	5.34±0.18 *n=60*	**0.027**
PCr/Pi	3.42±0.09‡,§ *n=61*	3.46±0.07$^{\P,\#}$ *n=62*	3.76±0.14 *n=61*	3.76±0.12 *n=60*	**0.009**
total ATP	42.58±0.38†,‡,§ *n=62*	40.06±0.33$^{\P,\#}$ *n=62*	45.24±0.38 *n=62*	44.21±0.41 *n=62*	**<.001**
NAD(H)	4.39±0.12† *n=49*	3.47±0.11$^{\P,\#}$ *n=61*	4.17±0.16 *n=45*	4.08±0.14 *n=44*	**<.001**
PEth	8.45±0.18† *n=61*	7.81±0.12$^{\P,\#}$ *n=62*	8.31±0.15 *n=60*	8.46±0.18 *n=58*	**<.001**
PCh	3.99±0.10 *n=55*	3.96±0.10 *n=61*	3.94±0.09 *n=56*	3.76±0.11 *n=54*	0.429
GPEth	4.76±0.15†,§ *n=51*	3.72±0.09$^{\P,\#}$ *n=61*	4.42±0.13 *n=57*	4.36±0.11 *n=57*	**<.001**
GPCh	6.69±0.26† *n=46*	5.01±0.12$^{\P,\#}$ *n=61*	6.11±0.15 *n=56*	6.28±0.16 *n=54*	**<.001**
pH	7.023±0.004‡,§ *n=61*	7.017±0.002$^{\P,\#}$ *n=62*	7.039±0.004 *n=61*	7.050±0.005 *n=60*	**<.001**

LS means ± SEM of metabolite levels, as percentage of total phosphorus signal, in anterior cingulate cortex, retrosplenial cortex and hippocampi. Significant ($p<0.05$) main effect of brain region indicated in bold. *PCr also has a significant interaction between group and brain region. $p<0.05$, ACC vs †RSC, ‡HL, or §HR; RSC vs ¶HL, or #HR. LS, least squares; SEM, standard error of the mean; n, number of observations; ACC, anterior cingulate cortex; RSC, retrosplenial cortex; HL, left hippocampus; HR, right hippocampus; PCr, Phosphocreatine; Pi, inorganic phosphate; ATP, adenosine triphosphate; NAD(H), nicotinamide adenine dinucleotide; PEth, phosphoethanolamine; PCh, phosphocholine; GPEth, glycerophosphoethanolamine; GPCh, glycerophosphocholine.

a rather drastic decrease in PCr's T1 value from about 3 to 2 seconds. Moreover, no increases in PME and PDE signals were detected, while those would be affected more severely by a T1 decrease[31]. A potential cause for relaxation enhancement is a higher brain iron accumulation in AD patients[32], but this would increase the signal linewidth, which was not observed. Thus, the most likely explanation for the increased PCr signal is a higher tissue concentration of this compound.

Elevated PCr signals have been reported before for AD patients in comparison with young controls[23], but as PCr also increases with age in healthy persons[30,33,34] it is unclear whether this finding was due to an aging effect. The current results showed increased PCr, and PCr/Pi, in AD patients compared with elderly controls. In contrast, two earlier studies showed a decrease in

prefrontal PCr in mild, but not severe, AD[24] and decreased PCr/Pi in the frontal lobe[21]. However, these studies were performed at 1.5T with only surface coil localization and involved small sample sizes.

As the concentration of high energy phosphates depends on the balance between energy production and energy utilization, increases in steady state PCr levels and PCr/Pi reflect a redistribution in the content of metabolites involved in the connected CK and ATPase pseudo-equilibria[35]. This may be the consequence of reduced utilization of ATP[33], caused by synaptic dysfunction or loss of other energy requiring functionalities[36,37]. Although these changes are expected to lower ATP production and thus PCr, the final balance between reduced energy production and consumption may still result in a higher PCr level. To maintain equilibrium, the increased PCr should be accompanied by changes in the levels of other substrates of the CK reaction, such as a higher ATP concentration. Previous studies reported an increase in (γ) ATP[23,24,38], although no alterations in ATP levels were found in the current nor in several other studies e.g. [21,22,39]. Alternatively, a higher PCr may result from an increased total amount of cellular creatine (Cr). However, *in vivo* measurement of total Cr content in MCI and AD patients provide no clear evidence that Cr is increased in the disease[40,41]. Finally, decreases in ADP and/or H^+ concentration may accompany the PCr increase. Indeed, a slight increase in pH was detected in mild AD patients, but it does not fully compensate for the increase in PCr when equilibrium is assumed. Changes in PCr levels have also been associated with decreased CK activity in the AD brain[23,33]. However, it is unlikely to be a direct effect of this decrease as even in cytosolic CK knockout mice the remaining CK activity ($< 20\%$) is sufficient to preserve equilibrium[42]. Clearly, a strongly affected reaction rate will affect processes that require rapid (local) energy supply, and a higher PCr level may be a flux adaptation to this condition.

There is some evidence for involvement of PCr in processes beyond the PCr-CK system. Since glutamate uptake by synaptic vesicles is stimulated by PCr, independent of ATP or CK[43], increased PCr may prevent glutamate excitotoxicity[44]. Furthermore, PCr may be able to bind to and stabilize phospholipid membranes[45] and may function as an osmolyte[46] and a neurotransmitter[47]. In addition, differences in metabolite levels may be explained by variations in tissue and cell type[48,49], although our results remained unchanged when adjusting for grey and white matter. A prominent neuropathological feature in AD is astrogliosis[50]. As this alters the neuron-to-glia ratio within grey and white matter[51], it is possible that morphological differences between AD patients and healthy aged controls play a role in the alterations in PCr and pH.

Contrary to our expectation, we have not observed differences between mild AD patients and controls in any of the phospholipid metabolites, although preclinical and post-mortem studies show alterations in membrane function and lipid composition in AD[7,20]. No previous *in vivo* studies have reported on PEth, PCh, GPEth or GPCh, but several studies reported changes of their total amounts, i.e. PME, PDE or their ratio, in AD[38,39,52], although others reported no

differences in these metabolites [21,53-55]. The most consistent finding seems to be an increase in total PME in the prefrontal cortex in AD compared with elderly controls[24,52,56]. In the present study, part of the frontal cortex was covered in the ACC measurement, but we did not find changes in PEth, PCh, nor total PME. However, changes in the complex phospholipid cycle may be too small at this disease stage to be detected with the current methodology. Although we had sufficient spectral resolution to resolve the phosphomonoester signal of PEth and PCh, and the phosphodiesters signal of GPEth and GPCh, these may still reflect metabolites connected to different phospholipid pools. Moreover, differential expression of phosphatidylethanolamine and phosphatidylcholine species across hippocampal subfields was recently shown in AD[57].

MRSI revealed that high energy phosphates, pH and most phospholipid metabolites vary extensively across brain regions that are of interest in AD. These differences existed in both mild AD patients and controls, and were not explained by regional variation in grey and white matter content, with the ratio PCr/Pi as the sole exception. This indicates that the investigated regions are metabolically different from each other. It also underlines that regional differences should be taken into account in any cross-sectional or longitudinal metabolic study. Moreover, as shown here for PCr, changes in metabolite levels in disease may not be the same for all brain regions.

The current study only reports on relative metabolite levels, as obtaining absolute quantifications requires several assumptions, which adds complexity and reduces reliability. Moreover, when the variation in atrophy between and within groups is large, relative measurements are preferred over absolute. In future studies it would be valuable to include preclinical AD patients or an at-risk population to clarify the disease stage in which abnormalities in PCr and pH appear. In addition, magnetization transfer experiments to determine the CK and ATPase reaction rates with measurements of absolute concentrations of PCr and ATP to determine fluxes[35], would elucidate whether the dynamics of the PCr-CK and ATPases systems are affected in AD.

Conclusion

To the best of our knowledge, this is the largest study to date investigating brain phospholipid and energy metabolism in AD by ^{31}P MRS. Increased PCr levels were found in regions that show early degeneration in AD, but not in the ACC, a region known to be involved in this disease at a later stage. Together with an increased pH, this indicates that energy metabolism is altered in mild AD. Although most phospholipid metabolites, like the high energy phosphates, differed between the investigated brain regions in both groups, no alterations in phospholipid metabolism were found specific to mild AD.

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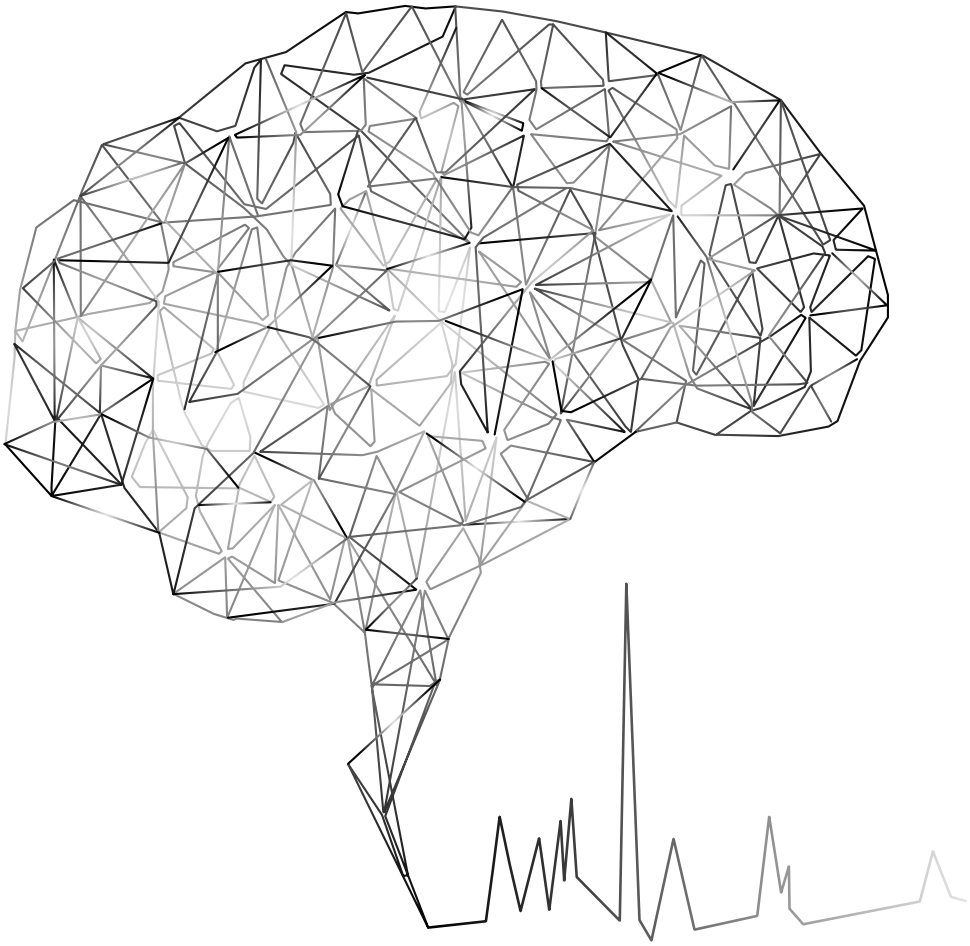
4

The medical food Souvenaid affects brain phospholipid metabolism in mild Alzheimer's disease



Anne Rijpma, Marinette van der Graaf, Marieke M. Lansbergen, Olga Meulenbroek, Aysun Cetinyurek-Yavuz, John W. Sijben, Arend Heerschap, Marcel G.M. Olde Rikkert. The medical food Souvenaid affects brain phospholipid metabolism in mild Alzheimer's disease

Submitted.

Abstract

INTRODUCTION: Synaptic dysfunction contributes to cognitive impairment in Alzheimer's disease (AD) and may be countered by increased intake of nutrients that target brain phospholipid metabolism. This study explores whether the medical food Souvenaid affects brain phospholipid metabolism in AD patients. METHODS: 34 drug-naïve mild AD patients (MMSE≥20) were enrolled in this exploratory double-blind randomized controlled study. Before and after 4 weeks intervention with Souvenaid or control product, phosphorus and proton magnetic resonance spectroscopy (MRS) assessed surrogate measures of phospholipid synthesis and breakdown (phosphomonoesters [PME] and phosphodiesters [PDE]), neural integrity (N-acetyl aspartate), gliosis (myo-Inositol) and choline metabolism (choline-containing compounds [tCho]). RESULTS: MRS data from 33 patients were analyzed. PME/PDE and tCho were higher after 4 weeks of Souvenaid compared with control. No differences were observed in the other MRS outcome parameters. DISCUSSION: Phosphorus MRS reveals that Souvenaid affects brain phospholipid metabolism in mild AD, in line with findings from preclinical studies.

Introduction

Synaptic dysfunction is a major contributing factor to cognitive impairment in Alzheimer's disease (AD)[1,2] and may be caused by deficits in neuronal membrane composition and function[3,4]. As the neuronal membrane is mainly composed of phospholipids[5], interventions that target brain phospholipid metabolism may affect cognitive function in AD.

The most abundant phospholipids in the neuronal membrane are phosphatidylethanolamine (PE) and phosphatidylcholine (PC)[4,5]. They are formed in the Kennedy cycle, wherein phosphomonoesters (PME) are converted to phospholipids that can then be incorporated in neuronal membranes[6]. The breakdown of these phospholipids releases phosphodiesters (PDE), that can either be used for resynthesis or be further broken down[7]. The synthesis of brain phospholipids is influenced by the availability of specific nutrients, consisting of rate-limiting phospholipid precursors, to the brain[8,9]. This is not only affected by nutritional intake, but also by the intake of cofactors that influence precursor uptake and metabolism. Increasing the availability of several precursors proved to have a synergistic effect on phospholipid formation as well as on dendritic spine density[9-12]. Furthermore, circulating levels of most of these nutrients (precursors and cofactors) as well as brain choline levels are lower in patients with AD[13-15], thus lowering precursor availability. The specific multi-nutrient combination Fortasyn® Connect (FC) in the medical food Souvenaid® contains precursors and cofactors in the phospholipid synthesis pathway (docosahexaenoic acid [DHA], eicosapentaenoic acid [EPA], uridine monophosphate [UMP], choline, phospholipids, selenium, folic acid, and vitamins B6, B12, C and E), and has been formulated to promote neuronal membrane (phospholipid) formation and function in AD[8]. Previous studies in animal models of AD and aging have shown that long term supplementation with FC positively affects explorative behavior and memory[16,17], in addition to enhancing phospholipid synthesis and improving cholinergic transmission[18]. This indicates that supporting synaptic function by increasing phospholipid formation is a promising strategy to improve cognition or reduce cognitive decline.

Randomized controlled trials in patients with AD demonstrated improvement in memory performance in mild AD over 12-24 weeks intervention with this specific multi-nutrient combination[19-21] as well as altered functional connectivity and brain network organization[22]. However, the physiological underpinnings in humans remain to be further elucidated.

Neither synapse number nor phospholipid membrane composition can be assessed directly *in vivo*, but phosphorus magnetic resonance spectroscopy (^{31}P-MRS) allows for the non-invasive investigation of phospholipid building blocks and breakdown products, i.e. PME and PDE, respectively[23]. In addition, using proton (^{1}H) MRS, brain metabolites related to neural integrity (N-acetyl-aspartate [NAA]), gliosis (myo-inositol [mI]), choline metabolism (total choline [tCho]) and energy metabolism (total creatine [tCr]) can be assessed as well[23,24]. Hence, the current study explores whether the medical food Souvenaid affects brain phospholipid metabolism and neural integrity in patients with mild AD using ^{31}P and ^{1}H-MRS.

Methods

Subjects and design

All visits took place between October 2012 and February 2015 at the Radboud university medical center (Nijmegen, the Netherlands). AD drug-naïve patients aged ≥ 50 years with a diagnosis of probable or possible AD, and a minimum Mini Mental State Examination (MMSE) score of 20, were recruited from the hospital's memory clinic or by referral from regional hospitals. All subjects were screened for magnetic resonance imaging (MRI) contra-indications before inclusion in the study. Written informed consent was obtained from all patients and their informal caregivers. The local ethics committee reviewed and approved the protocol. The study was conducted in accordance with the Declaration of Helsinki and registered in the Dutch Trial Register (NTR3346).

Eligible subjects were randomly allocated to receive either the test product (i.e. Souvenaid, containing the specific nutrient combination FC) or an iso-caloric control product once daily as a drink for a double-blind period of 4 weeks (1:1 randomization, stratified based on gender). The test product contained DHA, EPA, phospholipids, choline, UMP, selenium, folic acid and vitamins B6, B12, C and E (Supplementary Table A.1).

At baseline and after 4 weeks, venous blood samples were taken and MR measurements were performed. MR measurements at week 4 took place at least two hours after intake of the last study product. For the purpose of compliance and safety, a phone call was conducted after 14 days of product intake. A final follow-up call was conducted 2 weeks after the last visit. More details on subjects, study design, biochemical analyses of blood samples, the MR protocol and MR analyses can be found in the Supplementary Material.

MR protocol

MRI and MRS were performed on a Magnetom Tim Trio 3T MR system (Siemens, Erlangen, Germany) with a dual-tuned $^{1}H/^{31}P$ volume head coil (Rapid Biomedical, Wuerzburg, Germany). High resolution MR images were acquired with a T1-weighted magnetisation-prepared rapid gradient-echo sequence. ^{31}P-MR spectra were acquired with a whole brain 3D MR spectroscopic imaging (MRSI) pulse-acquire sequence. ^{1}H-MR spectra were acquired with a single slice 2D semi Localization by Adiabatic Selective Refocusing MRSI sequence (covering the hippocampi) or a single voxel point resolved spectroscopy sequence (anterior and posterior cingulate cortex [ACC and PCC]).

MR data analysis

Four regions of interest (ROI) were selected for analysis from the 3D ^{31}P-MRSI data: ACC, retrosplenial cortex (RSC), and left and right hippocampus (HL and HR). From the 2D ^{1}H-MRSI data, two voxels from the hippocampi (HL and HR, respectively) were selected for analysis, in addition to the single voxel data from the ACC and PCC (Figure 1AC).

The software packages Metabolite Report (Siemens Healthcare, Erlangen, Germany) and LCModel (Version 6.3-0C[25]) were used for post-processing and automatic fitting of ^{31}P-MR and ^{1}H-MR spectra, respectively. Both a quantitative evaluation of the fitting results and a visual quality control were performed.

The intensity of each phosphorus metabolite resonance was expressed as a percentage area of the total phosphorous signal area. PME was calculated as the sum of phosphoethanolamine (PEth) and phosphocholine (PCh), PDE as the sum of glycerophosphoethanolamine (GPEth) and glycerophosphocholine (GPCh), and total adenosine triphosphate (tATP) as the sum of α-ATP, β-ATP and γ-ATP. In addition, the ratios PME/PDE, PEth/GPEth, PCh/GPCh and phosphocreatine/inorganic phosphate (PCr/Pi) were computed.

Regarding ^{1}H-MRS, the intensities of *N*-acetylaspartate (NAA), *myo*-inositol and glycine (mI), and choline-containing compounds (total choline, tCho: PCh, GPCh and free choline) were expressed relative to the intensity of creatine and phosphocreatine (total creatine, tCr). Additionally, water-referenced levels of NAA, mI, tCho and tCr were expressed in m*M*, and the ratio of NAA to mI (NAA/mI) was calculated.

Statistical analyses

All statistical analyses were performed for the modified intention to treat (ITT) data set, including all subjects who received at least one unit of the study product and with at least one MR measurement. The main statistical analyses of the MRS outcome parameters were also performed for the per protocol (PP) population, which includes all subjects who had no major protocol deviations.

All blood outcome parameters at week 4 were analyzed using analyses of covariance (ANCOVA) with between-subjects factors intervention group and gender, and baseline measure as covariate.

All MRS outcome parameters at week 4 were analyzed using a predefined multilevel model with intervention group (test or control), gender, brain region and its interaction with intervention group as fixed factors, considering brain region as a within subject factor, and adjusting for baseline. The analyses were conducted (a) using the observed baseline as covariate (missing baseline values were set to missing, 'main model – observed') and (b) using the imputed baseline values (missing baseline values were replaced by imputed baseline values) as covariate ('main model – with imputation'). Imputation of the baseline value was performed by a regression imputation using age, gender, and brain region. An unstructured variance-covariance matrix for brain region was selected. If the p-value for intervention group by brain region interaction was <0.10 ANCOVA models per brain region were conducted. If the p-value for the intervention by brain region interaction was > 0.10, the interaction term was dropped from the model.

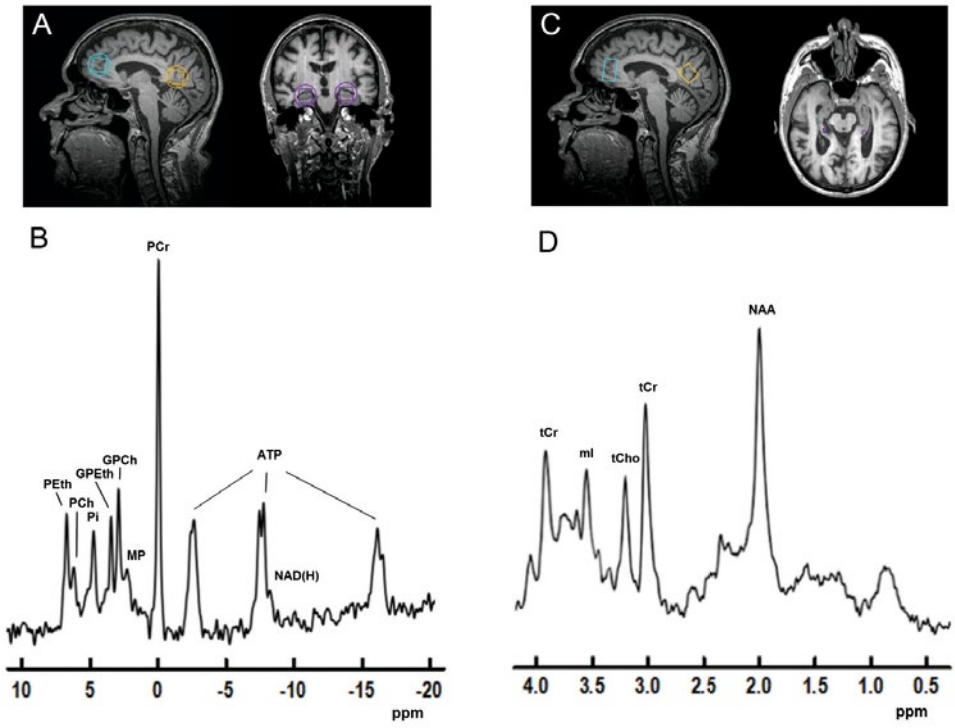


Figure 1

Voxel selection and representative examples of ^{31}P and ^{1}H MR spectra. A, voxel selection of ^{31}P MR spectra displayed on sagittal (left) and coronal (right) anatomical images: anterior cingulate cortex (blue), retrosplenial cortex (yellow), left and right hippocampus (purple). The nominal voxel size is indicated by squares and the approximation of the effective spherical voxel size is indicated by circles. B, representative ^{31}P MR spectrum from the RSC. Zero filling to 4096 data points and an 8 Hz Gaussian filter were applied. C, sagittal (left) and transversal (right) anatomical images showing single voxel volumina of ^{1}H MR spectra in anterior cingulate cortex (blue), posterior cingulate cortex (yellow), and voxel selection of left and right hippocampus (purple). D, representative ^{1}H MR spectrum from the PCC. Zero filling to 8192 data points and an 2 Hz Lorentzian filter were applied. PEth, phosphoethanolamine; PCh, phosphocholine; Pi, inorganic phosphate; GPEth, glycerophosphoethanolamine; GPCh, glycerophosphocholine; MP, membrane phospholipids; PCr, Phosphocreatine; ATP, adenosine triphosphate; NAD(H), nicotinamide adenine dinucleotide; ppm, parts per million; NAA, *N*-acetyl-aspartate; tCho, Choline-containing compounds; tCr, total Creatine; ml, *myo*-inositol.

Predefined supportive analyses involved linear mixed model analyses including the baseline value of the outcome parameter in the outcome vector and including intervention group, gender, time, brain region and 2-way and 3-way interactions between intervention group, brain region, and time as fixed factors, and a random intercept for time per subject. In this supportive model, the intervention effect is expressed by the interaction of time by intervention group. Additionally, the primary model analyses were repeated (a) with adjustments for pre-specified possible confounders (i.e., MMSE, education level [low, medium, high], gray matter fraction [GMf], and

intake of choline containing food [only for choline related outcome parameters]), and (b) for the one brain region for which most data was available. For all fitted models, the influence diagnostics were used to explore the influence of different observations on the models.

The supportive and covariate analyses were conducted only for the main ^{31}P-MRS (PME, PDE, PME/PDE) and main ^{1}H-MRS outcomes (NAA/tCr, mI/tCr, Cho/tCr, NAA/mI). Analyses of these main outcome parameters were performed on partially unblinded data (using intervention group coded as X and Y), allowing analysis of the intervention effect while keeping the statisticians blinded to group allocation. Statistical analyses were performed by both AR and AY using SAS 9.2 and SAS 9.4, respectively (SAS Institute Inc., Cary, NC, USA). Statistical significance for the intervention effect was set at $p < 0.05$ without correction for multiple testing.

Results

Subjects

Of the 40 subjects who were screened, 34 subjects were included and randomized to receive either the test or the control product. One subject dropped out from the study without MR measurements and prior to product dispense, due to unexpected claustrophobia which was an exclusion criteria for the current study as described in the study protocol. Hence, 33 subjects were included in the modified ITT population. Major protocol deviations were present in four subjects: no MR measurements at week 4 (n=2) or (suspected) double product intake prior to week 4 measurements (n=2). Hence, 29 subjects were included in the PP population (for a flowchart, see Supplementary Figure A.1). Subjects in the test (n=16) and control (n=17) groups (modified ITT population) were comparable with respect to baseline characteristics (Table 1). The test group reported 80 medical conditions in the medical history compared to 120 medical conditions in the control group. Adherence to the study product, according to diary entries, was high (>96%) and equal in both groups.

Safety analysis and concomitant medication

In total 27 adverse events (AEs) were reported, of which no serious AEs. The number of subjects with at least one AE did not differ statistically between groups (test: 13 AEs in 8 subjects, control: 14 AEs in 7 subjects, Fisher's exact test p=0.732). A total of 15 of 33 subjects (45.5% of the total study population) reported at least one AE. AEs that were most often reported for both study groups concerned gastro-intestinal system disorders (i.e. abdominal pain, diarrhoea, dyspepsia, nausea, vomiting). The majority of AEs were considered to be unrelated to the study product. Seven AEs (5 in the test group and 2 in the control group) had a relationship to the study product ('possibly' or 'probably'), and were all of gastro-intestinal nature (i.e. diarrhoea, dyspepsia, nausea, and vomiting). Regarding the use of concomitant medication (other than AD medication), 62.5% of subjects in the test group and 82.4% of the control group used

Table 1 Subject characteristics

	Test n=16	**Control n=17**
Age	74.7 years (4.8)	72.7 years (8.2)
Gender (%)	7 males (44%)	7 males (41%)
MMSE	23.0 (2.1)	23.2 (1.8)
Time since diagnosis	1.7 months [0.4-15.3]	1.4 months [0.2-10.2]
BMI	24.2 kg/m^2 (3.8)	26.7 kg/m^2 (3.3)
Educational level (%)		
- Low	6 (37.5%)	4 (23.5%)
- Medium	8 (50.0%)	9 (52.9%)
- High	2 (12.5%)	4 (23.5%)
Study product compliance	96.4% (4.8)	99.0% (1.6)

MMSE, mini mental state examination; AD, Alzheimer's disease; BMI, body mass index. Data are presented as mean (standard deviation) or median [range] unless otherwise indicated.

concomitant medication until the four weeks intervention period. After the intervention period, 4 of 16 subjects in the test group and 8 of 17 subjects in the control group started AD medication (galantamine, memantine, or rivastigmine).

Nutritional blood markers

Levels of uridine, choline, and vitamin E in plasma, and percentages of DHA, EPA, and total long-chain polyunsaturated fatty acids (LCPUFAs) in plasma as well as in total fatty acids in erythrocyte membrane were higher at week 4 in the test group compared with the control group (all $p<0.001$). Homocysteine levels were lower after 4 weeks of test product compared with the control product ($p=0.006$). Docosapentaenoic acid in plasma or erythrocyte membrane was not significantly different at week 4 between the groups ($p=0.628$ and $p=0.840$, respectively). For details see Table A.2.

MRS outcomes

Good quality ^{31}P and ^{1}H-MR spectra were obtained from all ROIs (Figure 1B and 1D). If no significant interactions between intervention group and brain region were observed in the statistical analyses, only main intervention group effects are reported. Furthermore, the results from the covariate analyses are only reported if the potential confounders substantially changed the intervention effect. As the ITT and PP analyses yielded the same findings, only the ITT analyses are reported.

Primary ^{31}P-MRS outcomes

A significant intervention effect, showing higher PME/PDE at week 4 in the test group than in the control group, was found using the main model with observed baseline (Least Square (LS) mean±SEM test: 1.35±0.06, control: 1.17±0.06, p=0.005), but not using the main model with imputed baseline (p=0.295) (Table 2). Influential diagnostics revealed an influential subject that severely affected the results of the model. Excluding this subject from the model, resulted in a similar effect of the intervention on PME/PDE at week 4 to the model with observed baseline (LS mean±SEM test: 1.29±0.06; control: 1.16±0.05, p=0.061, Table 2 Model A). Results from the supportive models aligned with the main modeling approach using observed baseline (for details, see Table 2, Model B-C). The supportive model D resulted in a significant intervention group by time interaction, which expresses the intervention effect (for details, see Table 2, Model D). No differences between groups were observed regarding the levels of PME (all models p>0.05, e.g. main model - with imputation LS mean±SEM at week 4, test: 12.04±0.19; control: 11.99±0.19, p=0.845) or regarding levels of PDE (all models p>0.05, e.g. main model - with imputation LS mean±SEM at week 4, test: 9.94±0.35; control: 10.41±0.35, p=0.329).

Other ^{31}P MRS outcomes

The ratio PEth/GPEth was higher in the test group than in the control group (only main model - observed baseline, LS mean±SEM test: 2.02±0.09; control: 1.80±0.09, p=0.091) (Table 3). There were no significant differences between groups regarding the ratio PCh/GPCh, levels of PEth, PCh, GPEth, GPCh, PCr, Pi, ATP, or nicotinamide adenine dinucleotide, or pH (all p>0.05).

^{1}H-MRS outcomes

Using the main model with observed baseline, a significant interaction with brain region was found for tCho/tCr, leading to analyses per brain region. These showed that tCho/tCr at week 4 was higher in the test group than in the control group in the ACC (LS mean±SEM test: 0.302±0.015; control: 0.258±0.016, p=0.068) and in the HR (LS mean±SEM test: 0.287±0.004; control: 0.264±0.004, p=0.003), but not in the HL and PCC (both p>0.05) (Table 4). Using the main model with imputed baseline, an overall significant intervention effect was found on relative tCho levels indicating that tCho/tCr at week 4 was higher in the test group than in the control group (Table 4; LS mean±SEM test: 0.264±0.004; control: 0.252±0.004, p=0.019).

In the supportive model of tCho/tCr, the p-value of the three-way interaction between intervention, brain region and time was below the pre-specified threshold (p<0.10), leading to per brain region analyses. The intervention had no significant interaction with time at any of the brain regions (all p>0.05). For absolute levels of tCho, both main models yielded a significant intervention effect indicating that levels of tCho were higher at week 4 in the test group than in the control group (LS mean±SEM test: 1.95±0.05 and 1.94±0.05; control: 1.84±0.05 and 1.83±0.05, p=0.007 and p=0.018; main model with imputed baseline and observed baseline, respectively)

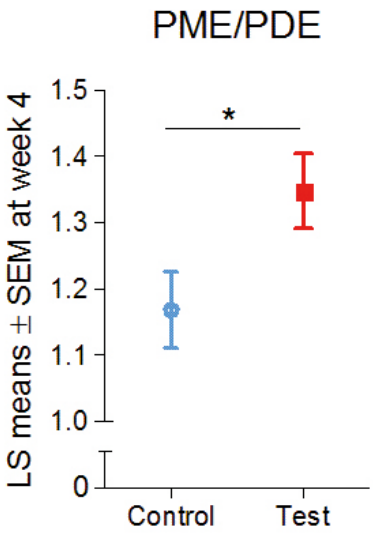


Figure 2

LS means ± SEM of PME/PDE at week 4 in the test and control group from the main model - observed baseline. LS, least square; SEM, standard error of the mean; PME, phosphomonoesters; PDE, phosphodiesters. * = $p<0.05$.

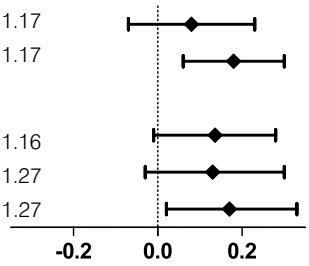
There were no significant differences between groups regarding relative (i.e. relative to tCr) or absolute (i.e. water-referenced) levels of NAA or mI, on the ratio NAA/mI or regarding absolute levels of tCre, in any of the modeling approaches (all $p>0.05$).

Discussion

This study investigated whether the medical food Souvenaid, containing nutritional precursors and cofactors for phospholipid membrane formation (i.e. FC), influenced brain phospholipid metabolism in mild AD. The observed effects indicate that this specific multi-nutrient combination not only raises circulating levels of phospholipid precursors after 4 weeks, but also affects the balance between brain metabolites of phospholipid formation and breakdown in patients with mild AD. In addition, levels of tCho were higher after the intervention in comparison with the control product, whereas metabolic measures of neural integrity, gliosis, and energy metabolism were not significantly affected.

Table 2 Results from statistical analysis for PME/PDE; ITT population.

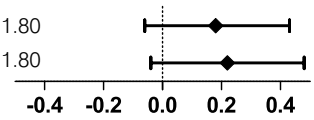
	N Test / N Control	Mean estimated value at week 4		Mean estimated group difference at week 4 (95% CI)	p-value
		Test	Control		
Main modeling approach					
Imputed BL	15/16	1.25	1.17	0.08 (-0.07,0.23)	0.295
Observed BL	14/15	1.35	1.17	0.18 (0.06,0.30)	0.005
Supportive models					
Model A	14/16	1.29	1.16	0.14 (-0.01,0.28)	0.061
Model B	15/16	1.40	1.27	0.13 (-0.03,0.30)	0.105
Model C	14/15	1.44	1.27	0.17 (0.02,0.33)	0.030
Model D	16/17	0.07*	-0.09*	0.16 (0.02,0.31)†	0.024#



Model A: Excluding influential subject, imputed BL; Model B: Most complete brain region (i.e. retrosplenial cortex), imputed BL; Model C: Most complete brain region (i.e. retrosplenial cortex), observed BL; Model D: Time included in model. Forest plots reflect mean estimated group differences at week 4 with 95% CI. PME, phosphomonoesters; PDE, phosphodiesters; ITT, intention to treat; CI, confidence interval; BL, baseline; na, not applicable. *Mean estimated change from baseline, †mean estimated group difference in change from baseline,#p-value for interaction between intervention and time (in days).

Table 3 Results from statistical analysis for PEth/GPEth; ITT population.

	N Test / N Control	Mean estimated value at week 4		Mean estimated group difference at week 4 (95% CI)	p-value
		Test	Control		
Main modeling approach					
Imputed BL	15/16	1.99	1.80	0.18 (-0.06,0.43)	0.135
Observed BL	15/15	2.02	1.80	0.22 (-0.04,0.48)	0.091

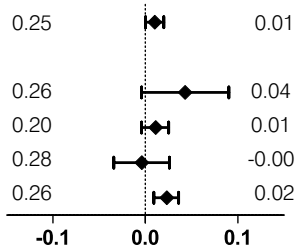


Forest plots reflect mean estimated group differences at week 4 with 95% CI. PEth, phosphoethanolamine; GPEth, glycerophosphoethanolamine; ITT, intention to treat; BL, baseline; CI, confidence interval.

There is strong *ex vivo* evidence that brain phospholipid content is decreased[3], phospholipid membrane composition is altered[3,26] and phospholipid anabolic and catabolic processes are disturbed[7,27] in AD and a tight link of these changes with synaptic loss and synaptic dysfunction is presumed[26,28]. The dependence of the unsaturated, low-affinity enzymes in the phospholipid synthesis pathway on substrate availability offers the opportunity to support this process by

Table 4 Results from statistical analysis for tCho; ITT population.

	N Test / N Control	Mean estimated value at week 4 Test	Control	Mean estimated group difference at week 4 (95% CI)	p-value
Relative tCho (tCho/tCr)					
Main modeling approach					
- Imputed BL	14/16	0.26	0.25	0.01 (0.00,0.02)	0.019
- Observed BL					
- ACC	10/8	0.30	0.26	0.04 (-0.00,0.09)	0.068
- PCC[1]	12/14	0.21	0.20	0.01 (-0.00,0.02)	0.139
- HL	9/11	0.28	0.28	-0.00 (-0.03,0.03)	0.794
- HR	9/10	0.29	0.26	0.02 (0.01,0.04)	0.003
Supportive modeling approach					
- Model D					
- ACC	14/11	-0.004*	-0.023*	0.019 (-0.047,0.085)[†]	0.578[#]
- PCC	14/16	0.006*	-0.004*	0.010 (-0.005,0.024)[†]	0.197[#]
- HL	12/15	-0.015*	-0.000*	-0.015 (-0.041,0.012)[†]	0.290[#]
- HR	12/14	0.003*	0.005*	-0.002 (-0.028,0.023)[†]	0.869[#]
Absolute (water-corrected) tCho (mM)					
Main modeling approach					
- Imputed BL	14/16	1.95	1.84	0.11 (0.03,0.19)	0.007
- Observed BL	14/16	1.94	1.83	0.11 (0.02,0.20)	0.018



Model D: Time included in model; [1]Supportive model: most complete brain region (posterior cingulate cortex), observed BL. Forest plots reflect mean estimated group differences at week 4 with 95% CI. ITT, intention to treat; CI, confidence interval; BL, baseline; na, not applicable. *Mean estimated change from baseline, [†]mean estimated group difference in change from baseline, [#]p-value for interaction between intervention and time (in days).

providing those substrates that are rate limiting. Short-term supplementation with this specific multi-nutrient combination increases plasma and erythrocyte levels of nutritional precursors and cofactors for phospholipid membrane formation in patients with mild AD, which confirms and extends previous studies[29], and thus may alleviate pre-existing nutritional deficiencies[14]. As several mechanisms have been described that move key nutrients across the blood brain barrier[9], the nutrients in the current investigation, or their metabolites, are expected to reach the brain. Accordingly, we observed a significantly increased ratio PME/PDE, which is considered to reflect the ratio of phospholipid anabolites over catabolites[23,30], across four brain regions with 4 weeks daily use of this multi-nutrient combination in mild AD. This indicates that the nutrients exert their effect on the brain's phospholipid metabolism, in line with the hypothesized mode of

action of this multi-nutrient combination. The observed changes in phospholipid metabolism may promote neuronal membrane formation, and may stimulate dendritic spine formation, as was shown previously in animal studies[8,9,11]. This may underlie the effects this intervention has on memory in patients with mild AD[19,20].

As the ratio PME/PDE changed significantly, but individual and total phosphomonoesters (PEth, PCh, and PME) and phosphodiesters (GPEth, GPCh, and PDE) did not, it cannot be established whether phospholipid formation was increased, breakdown was decreased, or both. In addition, the literature is inconclusive on *in vivo* levels of these metabolites in AD, since both increased, decreased and unaltered levels of PME and PDE have been observed in comparison with cognitively normal controls[31-36]. However, the effect of this multi-nutrient combination on brain phospholipid metabolism appears to be mainly driven by the ethanolamine pathway, as PEth/GPEth, but not PCh/GPCh, showed a trend towards an increase. This is in line with other studies that show that oral phospholipid precursors (pyrimidines and choline) in healthy populations[37-39] predominantly affect the ethanolamine pathway. However, in the current study, both relative and absolute tCho levels were higher at follow up in the group receiving the multi-nutrient intervention than in the control group, indicating an effect on phosphocholine metabolism as well. The tCho signal reflects the choline-containing compounds PCh, GPCh and free choline, and while both PCh and GPCh were measured separately with ^{31}P-MRS, no increase was detected in either one. This discrepancy may have arisen because ^{1}H and ^{31}P-MRS brain volumes were slightly different, but supportive statistical models could not confirm the significant alterations of tCho after 4 weeks intervention, warranting cautious interpretation. The analyses did reveal that changes in tCho may not be anatomically uniform, as most prominent effects were observed in the ACC and HR.

Reduced levels of NAA and increased levels of mI have been consistently shown in AD[40] and it might have been expected that this multi-nutrient combination would decelerate the changes in these measures of neural integrity and gliosis. Although uridine and DHA were found to stimulate neurite outgrowth[41,42], and DHA is known to have anti-inflammatory effects in addition to their role in the phospholipid synthesis pathway[43], no group differences after 4 weeks of intervention were observed regarding relative or absolute levels of NAA or mI, nor regarding the ratio NAA/mI. However, the normal rate of change over 4 weeks time may be limited, such that a deceleration effect of the intervention is not yet discernible, or it may be that the damage that these metabolites reflect is already irreversible at this stage of the disease.

The findings of this study may be limited by the modest sample size. Although there was sufficient power to detect an effect on the ratio PME/PDE, one of the main outcome measures, it was not possible to ascribe the increase in this ratio to increased PME or decreased PDE. Moreover, MR spectra were not always available or of sufficient quality from all investigated brain regions. Finally, a longer intervention duration may have led to more robust findings and more pronounced secondary outcomes.

This exploratory double-blind randomized controlled study showed that the medical food Souvenaid affects phospholipid metabolism across multiple brain regions in mild AD after only 4 weeks. This could lead to increased neuronal membrane formation, which would support the hypothesized mode of action of this multi-nutrient intervention. Larger and longer randomized controlled trials are needed to determine long term effects on phospholipid formation, synaptic function and cognition in persons with and at risk for AD.

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SUPPLEMENTARY MATERIAL

Methods

Subjects and design

Diagnosis of probable or possible AD was made according to the revised National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA) 2011 criteria[1] with evidence of the pathophysiological process (i.e. from structural MRI or cerebrospinal fluid biomarker assays). A Geriatric Depression Scale (GDS) score below 6 (out of 15) was required for inclusion. All subjects were AD drug-naïve (cholinesterase inhibitors and/or NMDA-antagonists) and were free from neurological or psychiatric disorders (other than dementia). To increase subject recruitment, eligibility criteria were extended during the study to include patients that were off AD medication for at least three months prior to the study, but no additional subjects were recruited that did not comply with the original criteria. Consumption of oily fish, more than twice a week, or of nutritional supplements containing docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA) was prohibited within two months prior to baseline. One month prior to baseline supplements containing more than 200% of the recommended daily allowance of vitamins B6 (2.8 mg), B12 (5 ug), C (160 mg), E (24 mg) or folic acid (0.4 mg) were prohibited, as well as anticholinergic and antipsychotic medication, and other medical foods or investigational products. Additionally, absence or stable doses of lipid lowering medications, antihypertensives and antidepressants in the month prior to baseline were required.

Medical history, medication and nutritional supplement use, Mini Mental State Examination, GDS, date of birth, sex, ethnicity, smoking habits, alcohol consumption, and family history of AD were recorded from all subjects at the first (screening) visit. Level of education was classified according to Verhage[2] (comparable with the International Standard Classification of Education[3]) and categorized in three groups: primary education or lower (low), junior vocational training (middle), and senior vocational or academic training (high).

In the randomization procedure two sets (one for women, one for men) of numbered and sealed randomization envelops were generated at Nutricia Research by the Clinical Studies Supplies Manager and opened upon randomization on-site by the investigator. Each envelop contained one of four codes, half representing the test group and half the control group. All subjects and any person involved in subject recruitment, group allocation, data acquisition and processing, or statistical analyses were blinded to the intervention group (test or control) until the analyses of the main outcome parameters were completed (see 2.4 for details on semi-blinded analyses). The composition of the test product is specified in supplementary Table A.1. The study product was packaged in tetra-packs (until 15 January 2014) or plastic bottles (from 15 January 2014 onwards) and labeled with one of the four randomization codes. With assistance from their

informal caregiver, subjects recorded intake of the product daily in a diary, which was used to verify intake at the week-4 visit.

Subjects were instructed to minimize intake of high-choline food on the days of the baseline and week 4 visits and to keep intake of concomitant nutritional supplements and medication stable (unless deemed necessary by their physician) during the study.

Table A.1 Nutritional composition of the study products; amount per daily dose (125 mL)

	Control	**Active**
Energy	125 kcal	125 kcal
Protein	3.8 g	3.8 g
Carbohydrate	16.5 g	16.5 g
Fat	4.9 g	4.9 g
EPA	0	300 mg
DHA	0	1200 mg
Phospholipids	0	106 mg
Choline	0	400 mg
UMP	0	625 mg
Vitamin E (alpha-TE)	0	40 mg
Vitamin C	0	80 mg
Selenium	0	60 μg
Vitamin B12	0	3 μg
Vitamin B6	0	1 mg
Folic acid	0	400 μg

EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; UMP, uridine monophosphate; TE, tocopherol equivalents * Test product is Souvenaid, containing the specific nutrient combination Fortasyn Connect. Souvenaid and Fortasyn are registered trademarks of Nutricia N.V.

Biochemical analyses of blood samples

Analyses of the blood samples were performed as reported previously[4]. A brief description is given here. The fatty acid composition of the total lipid fraction in plasma and erythrocytes was analyzed qualitatively on a gas chromatograph after extraction of the lipids from the plasma and/or erythrocytes and a methylation step. Plasma vitamin E levels were measured using high-performance liquid chromatography (HPLC) to determine the content of alphatocopherol. Homocysteine levels (free and protein-bound) were measured using HPLC with fluorescence detection after preparing a derivate. To determine plasma uridine level, perchloric acid was added to the sample. Uridine was extracted by vortexing the solution, followed by separation from other nucleotides and/or nucleosides using reversed-phase HPLC. The compounds

were quantified by measuring absorbance compared with a standard. HPLC electrochemical detection of plasma-free choline was performed according to a method adapted from Fossati et al.[5].

MR protocol

High resolution structural MR images were acquired with a T1-weighted magnetisation-prepared rapid gradient-echo sequence (TR=2300 ms, TE=3.16 ms, TI=1100 ms, 15° flip angle, 176 sagittal slices, slice-matrix size=256×256, slice thickness=1 mm, voxel-size=1×1×1mm, TA=6:25 min).

^{31}P-MR spectra were acquired with whole brain 3D MRSI (TR=2000 ms, 40° flip angle, acquisition delay=0.10 ms, WALTZ4 proton decoupling at 50% of 512 ms acquisition duration, FOV 260x260x260 mm; matrix size=10x10x10, acquisition time 13:08 min). K-space was sampled with a weighted elliptical phase encoding scheme with four averages. The field of view was centered on the midline and parallel to the line from the anterior commissure to the posterior commissure. Spatial post-processing consisted of zerofilling to a matrix size of 16x16x16 followed by spatial Fourier transformation. The nominal volume of the measured voxels is about 17.5 cc, the effective voxel at 64% of the spatial response function has a spherical shape with a volume of about 40 cc[6].

^{1}H-MR spectra of the hippocampus were acquired with a single slice 2D semi Localization by Adiabatic Selective Refocusing (sLASER) MRSI sequence, with water suppression enhanced through T1 effects (WET) (TR=2100 ms, TE=30 ms, 90° excitation flip angle, NA=6, nominal voxel size=3.75x5x10mm), positioned parallel to the hippocampi. ^{1}H-MR spectra of the anterior and posterior cingulate cortex (ACC and PCC) were acquired with a single voxel point resolved spectroscopy (PRESS) sequence with chemical shift selective water suppression (CHESS) (TR=3000 ms, TE=30 ms, 90° excitation flip angle, NA=64, voxel size=27x15x15 mm (ACC) and 20x20x16 mm (PCC)). With each sequence an additional MR spectrum without water suppression was obtained (2D SLASER: NA=1, PRESS: NA=8). The complete MR protocol, including positioning of the patient, took approximately 90 minutes. As the ^{31}P-MRS outcomes PME, PDE and PME/PDE were the main outcome measures, ^{31}P-MRS measurements were always performed before ^{1}H-MRS measurements, in case subjects failed to complete the entire scanning protocol.

^{31}P-MRS data analysis

Using the T1-weighted images the MRSI grid was shifted in the x, y and z dimension to position voxels in the ROI's. For the ACC, a voxel was selected anterior to the genu of the corpus callosum, with the inferior border aligned with the the line from the anterior commissure to the posterior commissure (AC-PC line), and on the midsagittal plane. For the retrosplenial cortex (RSC), a voxel on the midsagittal plane was selected posterior to the splenium of the corpus callosum,

with the inferior border aligned with the AC-PC line. For left and right hippocampus (HL and HR), a voxel was selected at the anterior end of each hippocampus, with the inferior and lateral border of the voxel aligned with the inferior and lateral side of the hippocampus.

The software package Metabolite Report (Siemens Healthcare, Erlangen, Germany) was used for post-processing (i.e. zero-filling, phase correction,100 ms exponential filter, baseline correction) and for automatic fitting of the spectra in the time-domain using prior knowledge appropriate for ^{31}P MR spectra (own data and de Graaf[7]). Eleven well-resolved resonance peaks were fitted: the phospholipid metabolites phosphoethanolamine (PEth), phosphocholine (PCh), glycerophosphoethanolamine (GPEth) and glycerophosphocholine (GPCh), the high-energy phosphorus molecules phosphocreatine (PCr), adenosine triphosphates (ATP), α-ATP, β-ATP and γ-ATP, nicotinamide adenine dinucleotide (NAD(H)) and inorganic phosphate (Pi), and membrane-bound phospholipid (MP). Regarding the quantitative evaluation of the fitting results, only metabolite fits with a Cramer–Rao lower bound (CRLB) of ≤30% were considered reliable. Qualitatively, two spectroscopists (AR and MvdG) independently checked the spectra by visual inspection of the original spectra and the fitting results. If a metabolite peak was visually present, and its fit was assigned to the correct resonance—giving a minimal residue—the fitting result was accepted. Since the intensity of each metabolite resonance was expressed as a percent area of the total phosphorous signal area., our data are corrected for CSF content and thus for differences in atrophy. Intracellular pH was determined from the chemical shift difference between the PCr and Pi resonance peaks[8].

^{1}H-MRS data analysis

The software package LCModel (Version 6.3-0C[9]) was used for post-processing (e.g. eddy current correction and water scaling) and for automatic fitting of all ^{1}H-MR spectra (i.e. 2 hippocampal voxels, ACC and PCC). Regarding the quantitative evaluation of the fitting results, a whole spectrum was rejected when the full-width-half-maximum >0.15 ppm (18.5 Hz) or the signal-to-noise ratio (S/N) <5, and only individual metabolite fits with a CRLB below 30% were considered reliable. Qualitatively, two spectroscopists (AR and MvdG) independently judged the original spectra and the fitting results by visual inspection according to a pre-specified set of criteria.

MRI data analyses

T_1-weighted MR images from one visit were segmented into grey matter (GM), white matter (WM) and cerebrospinal fluid (CSF) using automatic segmentation software (SPM8, Welcome Trust Centre for Neuroimaging, London, UK; VBM8, Structural Brain Mapping Group, Jena, USA). For each subject the whole brain GM fraction (GM / (GM + WM + CSF)) was calculated and used as a pre-specified confounder in supportive covariate analyses (see section 2.7).

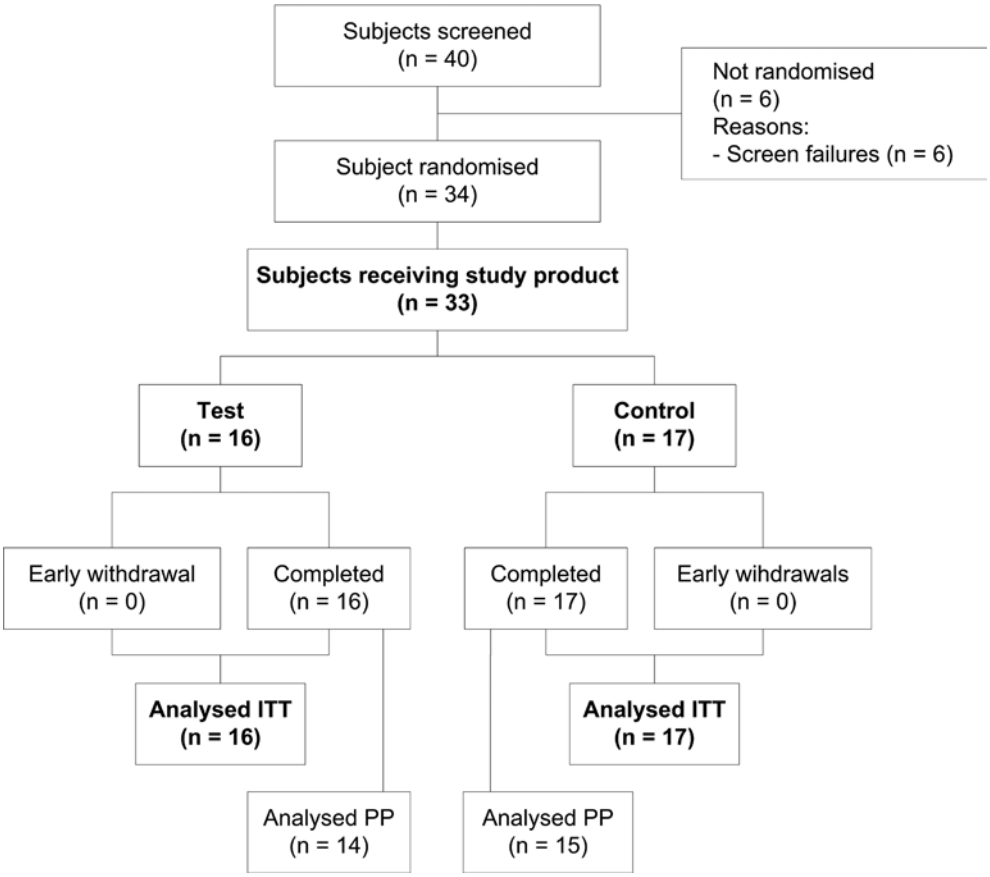

Figure A.1

Flowchart of subjects in study. ITT, intention to treat; PP, per protocol.

Table A.2 Intervention effect on nutritional blood markers and homocysteine.

Blood outcome parameter	Mean estimated value at week 4		Difference between groups at week 4	*p*-value for intervention effect
	Test n=16	Control n=17		
Fatty acids in the erythrocyte membrane				
DHA (%)	4.9	2.8	2.1 (1.84,2.30)	<0.001
EPA (%)	1.7	0.8	0.8 (0.69,0.98)	<0.001
DPA (%)	1.6	1.6	0.014 (-0.12,0.15)	0.840
LC-PUFA (=DHA+EPA+DPA) (%)	8.2	5.3	2.9 (2.51,3.24)	<0.001
Fatty acids in blood plasma				
DHA (%)	4.4	1.5	2.9 (2.59,3.12)	<0.001
EPA (%)	2.1	0.8	1.3 (1.10,1.51)	<0.001
DPA (%)	0.50	0.49	0.008 (-0.03,0.04)	0.628
LC-PUFA (=DHA+EPA+DPA) (%)	7.0	2.8	4.2 (3.79,4.53)	<0.001
Choline levels in blood plasma (uM)	14.4	9.0	5.4 (3.39,7.47)	<0.001
Vitamin E levels in blood plasma (uM)	50.9	39.6	11.4 (6.77,15.96)	<0.001
Homocysteine levels in blood plasma (uM)	9.8	12.4	-2.6 (-4.31,-0.82)	0.006
Uridine levels in blood plasma (uM)	14.8	2.6	12.2 (7.24,17.11)	<0.001

95% confidence interval (CI) is presented between brackets; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; DPA; docosapentaenoic acid; LC-PUFA, long-chain polyunsaturated fatty acids.

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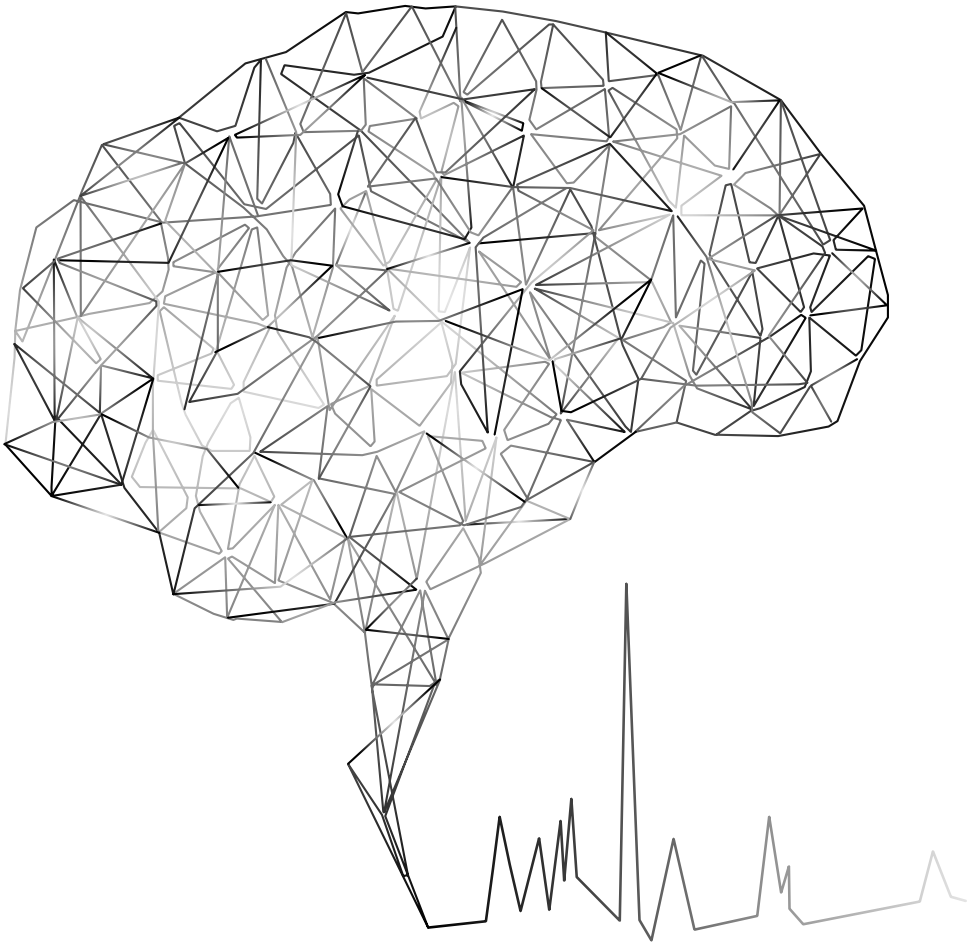
5

Cholinesterase inhibitors and add-on nutritional supplements in Alzheimer's disease. A systematic review of randomized controlled trials



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Ageing Research Reviews, 16, 105-112.

Abstract

To date, single drug and nutrient-based interventions have failed to show a clinically relevant effect on Alzheimer's disease (AD). Multidomain interventions may alleviate symptoms and alter the disease course in a synergistic manner. This systematic review examines the effect of adding nutritional supplementation to cholinesterase inhibitors. A systematic PubMed and Cochrane search resulted in nine high quality studies. The studies had low to moderate risk of bias and focused on oxidative stress, homocysteine levels, membrane fluidity, inflammation and acetylcholine levels. Only the use of vitamin E supplements could reduce the rate of functional decline when combined with cholinesterase inhibitors in one study, whereas cognition was not affected in both this and other studies. None of the other nutritional supplements showed convincing evidence of a beneficial effect when combined with cholinesterase inhibitors. This shows that cognitive and functional improvement is difficult to achieve in patients with AD, despite epidemiological data and evidence of biological effects of nutritional supplements. Addressing one disease pathway in addition to cholinesterase inhibitor therapy is probably insufficient to alter the course of the disease. Personalized, multifactorial interventions may be more successful in improving cognition and daily functioning.

Introduction

There is wide interest in the role nutrition plays in the development and disease process of neurodegenerative diseases. Epidemiological evidence suggests that certain diet patterns or dietary elements can prevent or slow the development of Alzheimer's disease (AD)[1]. In particular, low intake of omega-3 fatty acids, antioxidants and B-vitamins, have been associated with an increased risk of AD[2]. Moreover, brain structure and cognitive performance have been shown to be positively influenced by dietary nutrients, such as B-vitamins[3,4]. These findings raise the question whether supplementation with nutritional components may be of benefit to patients with Alzheimer's disease.

There are several disease pathways and risk factors in AD that could theoretically be affected by nutritional factors, such as oxidative stress, high homocysteine levels, reduced membrane fluidity, inflammation and low acetylcholine levels[5-9].

A proposed mechanism of oxidative stress is that activation of protein kinases, enhancing beta- and gamma-secretase activity, and lipid DNA and protein oxidation are induced by reactive oxygen species (ROS), leading to neuronal cell death[10,11]. The increased neurofibrillary tangle and amyloid-beta load in turn increase the amount of ROS. High homocysteine levels may lead to neurodegeneration by amyloid-beta peptide generation, hyperphosphorylation of tau or direct neurotoxic effects, or through its role in cerebrovascular pathology reviewed in [12]. Reduced membrane fluidity may lead to impaired neuronal communication, since that is dependent on proper functioning of membrane related mechanisms such as postsynaptic receptor functioning, and presynaptic fusion and endocytosis of vesicles[13]. Neuroinflammation, albeit most likely a secondary event, can cause or exacerbate neuronal death and is most prominent in those brain areas with high levels of AD pathology[7]. Finally, loss of cholinergic neurons in de nucleus basalis lead to actylcholine deficiencies and contribute to impaired memory in patients with AD[8].

Previous reviews have focused on nutrient status in patients with AD[14] or on the effect of single nutrients, such as vitamin E[15], or nutrient groups, such as B-vitamins[16] on cognition. To date, single nutrients have failed to show an indisputable, clinically significant effect on the severity or course of the disease, despite consistent epidemiological evidence of protective effects. This may be due to the heterogeneity of AD, which may be based on the multi-causal nature of the disease, thus suggesting that a multidimensional intervention is necessary to reach an improvement in cognition or daily function[17,18]. Treatments in which multiple elements are combined may have the potential to create a synergistic effect on the disease process, and interest in combination treatment has grown over the years. Therefore, this systematic review examines the effect of nutritional supplements combined with cholinesterase inhibitors on cognition and functional performances in patients with Alzheimer's disease.

Methods

This review has been reported according to guidelines of the Dutch Cochrane Centre[19] and meta-analyses (PRISMA) guidelines for reporting systematic reviews[20].

A comprehensive search strategy was developed to identify intervention trials which assessed the impact of cholinesterase inhibitors with nutritional supplements on Alzheimer's disease using both medical subject headings (MeSH) and key word terms. A complete listing of search terms is provided in the appendices (A, PubMed; and B, Cochrane Library). A preliminary search was performed in PubMed on January 16, 2012 and in the Cochrane Library on January 24, 2012; no language or date restrictions were applied in the search. However, studies in a language other than English were later excluded. E-mail notifications identifying new studies matching the search terms in PubMed were evaluated (AR) for eligible studies until May 17, 2013. Reference lists of all eligible studies were further cross-checked to identify additional trials.

Eligible studies included randomized controlled trials (RCTs) in which:
1) patients with Alzheimer's disease were included, (based on internationally accepted criteria);
2) the intervention consisted of one or more nutritional supplements;
3) the subjects where either given cholinesterase inhibitors as part of the intervention or were stable on cholinesterase inhibitors at the start of the study;
4) the population was aged 50 years or older.

Studies conducted in patients with major physical or cognitive disabilities (other than AD) and other types of dementia (e.g. Parkinson's disease dementia, Lewy Body dementia, vascular dementia) were excluded, as they were beyond the scope of this review. Studies in a language other than English were also excluded.

Risk of bias for the selected studies was independently assessed by two reviewers (OM and AR) using form II for RCT from the Evidence Based Guideline Development (EBRO; workgroup on guideline development, including the Dutch Cochrane Centre). After consensus was reached a judgment of low risk (+), unclear risk (?) or high risk (-) of bias was made on six types of bias: 1) 'Allocation' refers to the random allocation to treatment groups; 2) 'blinding´ refers to the degree of blinding that was applied (e.g. of participants or investigators); 3) 'incomplete outcome data' refers to loss to follow up; 4) 'selective reporting' refers to not reporting on all outcome measures; 5) 'comparable treatment groups' refers to whether there were any relevant differences between the treatment groups (e.g. age or age at onset of AD); 6) 'other bias' refers to other sources that could increase the risk of bias, such as carry-over effects in cross-over designs or conflicts of interest. Data on study and participant characteristics, supplement dose, method of cognitive assessment, and relevant outcomes were independently extracted by two reviewers (OM and AR). Where necessary, the corresponding author of the study was contacted to provide additional information.

Results

The electronic search to January 2012 resulted in a total of 479 results. After the removal of 32 duplicate citation, the 447 remaining citations were assessed for eligibility (Figure 1). Based on the title and/or abstract, 413 records were excluded, leaving 31 citations for which the fulltext articles were obtained. An additional six records were identified by cross-checking the reference lists from the 31 potentially eligible studies. Of these six records, four were immediately excluded based on the abstract. Thus, 33 full article citations were reviewed for eligibility. Following assessment by two authors (AR and OM), six articles describing five unique studies were included.

E-mail notification from PubMed up to April 8, 2014 resulted in the inclusion of four additional studies, resulting in a total of nine individual studies included in the final version of this review.

Risk of bias

The nine included studies had low to moderate risk of bias, as is shown in Figure 2. Random allocation and blinding of the patients and investigators to treatment group were well performed across most studies. However, the open-label study of Arlt et al.[21], was vulnerable to bias due to the randomization method and lack of blinding. The occurrence of incomplete outcome data could not be excluded in three out of nine studies[22-24]. Selective reporting could not be determined for all but one study[24]; little information was available on the placebo used and whether the reported outcome measures were the only ones used. For five out of nine studies, the treatment groups were comparable[23,25-28]. One study had a high risk of bias on this item, since treatments groups were not comparable with respect to age and age of onset of the disease[21] and the risk was unclear for two studies since no characteristics of subgroups were reported[24] or differences between groups were not formally tested[22]. There was generally low risk of other forms of bias, the only potential bias comes from Shah et al.[29] which was funded and co-authored by industry and Freund-Levi et al.[26], which was co-funded by industry.

Effect of nutritional supplements

The study characteristics and results of all nine studies are summarized in table 1. In case additional interventions were described that did not meet our inclusion criteria, we only evaluated and extracted the data that were relevant to the current study (e.g. the comparison between memantine and vitamin E[22] was not evaluated in this paper). Below the results of the studies are discussed grouped by nutritional compound and pathophysiological pathway connected to the supplements.

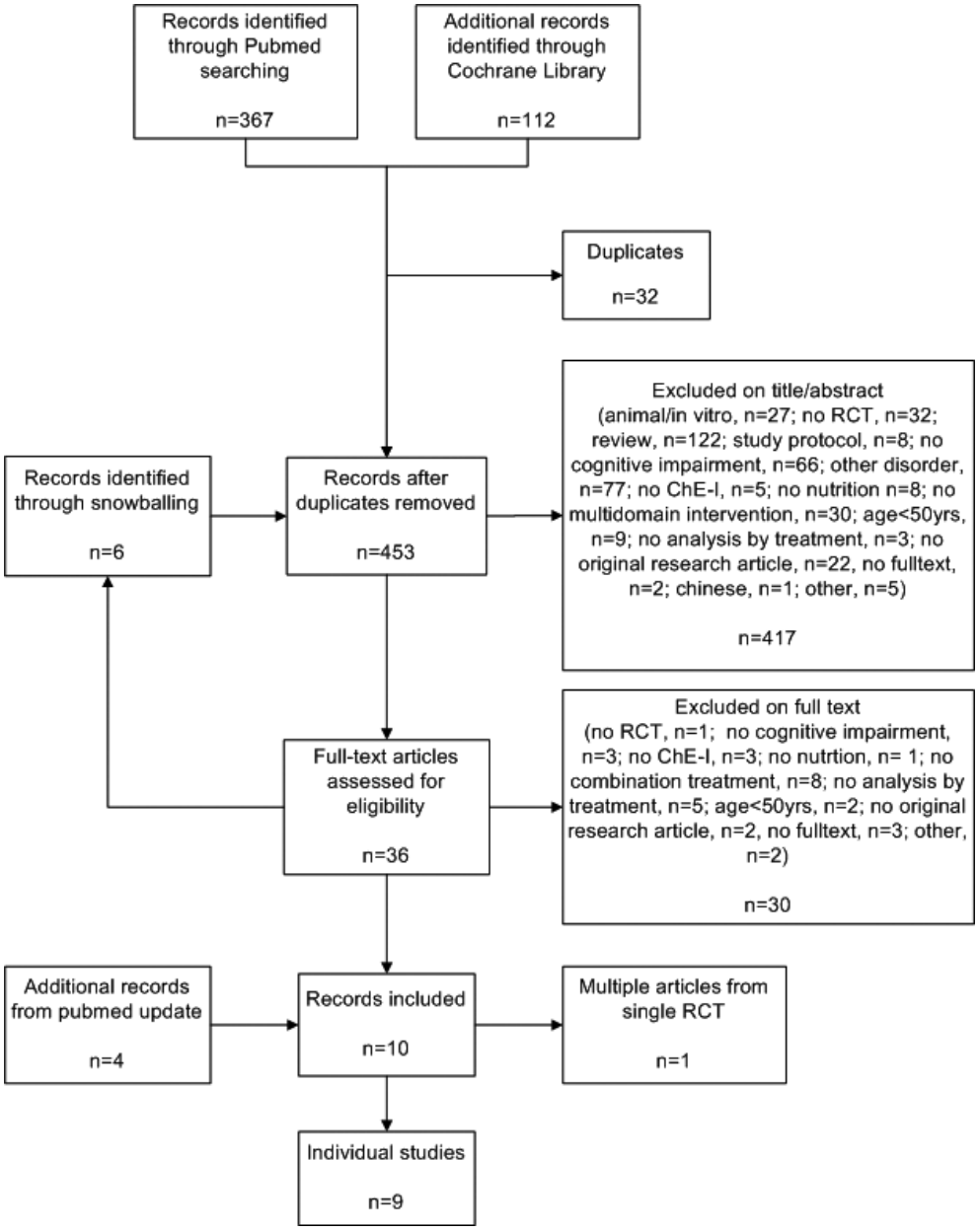

Figure 1

Flowchart of search process in PubMed and Cochrane library databases

Figure 2

Summary of bias of the included studies; + low risk, - high risk, ? unclear risk.

Table 1 Study characteristics and results.

first author, year	Population[$] (mean baseline MMSE score)	country	N (% female)	Mean age	Design	Acetyl-cholinesterase, daily dose	nutritional supplement, daily dose	duration of intervention
Amenta, 2012*	Mild to moderate AD with vascular damage (UK)	Italy	91 (64)	75	parallel group	donepezil, 10 mg	choline alphoscerate, 1200 mg; or placebo	12 m
Arlt, 2012	Mild to moderate AD (20.8)	Germany	23 (57)	71	parallel group	donepezil, 5-10 mg; or galantamine, 16 mg	vitamin C, 1000 mg + vitamin E, 400 IU; or placebo	12 m
Conelly, 2008	Mild AD (23.5)	Scotland / UK	41 (71)	76	parallel group	unknown	folic acid, 1 mg; or placebo	6 m
Cornelli, 2010	Mild AD (23.6)	USA	52 (60)	74	parallel group	donepezil, 5 mg	Formula F; or placebo	6 m
Dysken, 2014	Mild to moderate AD (21)	USA	304 (295)	79	parallel group	donepezil, galantamine or rivastigmine	vitamin E, 2000 IU	6-48 m
Foster, 1996	Mild to moderate AD (18)	USA	440 (62)	70	cross-over	tacrine, 40-80 mg	lecithin, 9040 mg; or placebo	0.9 m
Freund-Levi, 2006	Mild to moderate AD (23.4)	Sweden	204 (54)	74	parallel group	donepezil, galantamine or rivastigmine	omega-3, DHA 1720 mg and EPA 600 mg; or placebo	6 m
Shah, 2013	Mild to moderate AD (19.5)	USA	527 (253)	77	parallel group	unknown	Souvenaid; or placebo	5.5 m
Sun, 2007	Mild to moderate AD (18.7)	Taiwan	89 (49)	75	parallel group	donepezil	mecobalamin (vit B12), 0.5 mg; vit B6, 5 mg; folic acid, 5 mg + other vit and iron; or placebo	6 m

Table 1 *(continued)*

first author, year	outcome measures - cognition	baseline intervention (control)	intervention (control) change from baseline	p value	net change between groups	p value
Amenta, 2012*	MMSE	ND (ND)	-	NS (<.05)	-	ND
	ADAS-cog	ND (ND)	-	NS (<.05)	-	ND
Arlt, 2012	MMSE	20.0 (21.7)	-2.1 (-1.6)	<.05 (NS)	-0.5	NS
	Word List - immediate recall	2.6 (3.6)	0.6 (-0.7)	ND (ND)	+1.3	NS
	Word List - delayed recall	3.7 (3.3)	-0.4 (0.4)	ND (ND)	-0.8	NS
	word fluency	8.8 (9.4)	0.4 (-0.4)	ND (ND)	+0.8	NS
	TMT	66.1 (44.1)	-5.1 (9.8)	ND (ND)	-14.9	NS
Conelly, 2008	MMSE	23.48 (23.50)	0.09 (0.22)	ND (ND)	-0.13	NS
	DSST	21.35 (17.72)	3.26 (3.00)	ND (ND)	+0.26	NS
Cornelli, 2010	MMSE	23.2 (23.9)	1.1 (0.3)	ND (ND)	+0.8	ND
Dysken, 2014	MMSE	21.3 (20.8)	-2.97 (-3.16)	ND (ND)	+0.19	NS
	ADAS-cog	18.5 (19.1)	5.97 (7.78)	ND (ND)	-1.80	NS

Table 1 *(continued)*

first author, year	outcome measures - cognition	baseline intervention (control)	intervention (control) change from baseline	p value	net change between groups	p value
Foster, 1996	MMSE	17.9 (18.1)	0.8 (0.4)	NS (NS)	+0.4	NS
	ADAS-cog	26.1 (26.9)	-1.1 (-1.5)	NS (NS)	+0.4	NS
Freund-Levi, 2006	MMSE	23.6 (23.2)	-0.8 (-0.8)	NS (NS)	0.0	NS
	ADAS-cog	25.7 (27.2)	2.0 (1.1)	NS (NS)	+0.9	NS
Shah, 2013	ADAS-cog	23.89 (23.39)	1.88 (1.52)	ND (ND)	+0.36	NS
	Cognitive Test Battery	0.08 (-0.02)	-0.10 (-0.05)	ND (ND)	-0.05	NS
Sun, 2007	MMSE	18.7 (18.6)	0.15 (0.41)	ND (ND)	-0.26	NS
first author, year	**outcome measures - functional**	**baseline intervention (control)**	**Intervention (control) change from baseline**	**p value**	**net change between groups**	**p value**
Amenta, 2012*	BADL	ND (ND)	ND (ND)	NS (NS)		ND
	IADL	ND (ND)	ND (ND)	NS (<.05)		ND
Conelly, 2008	IADL (NOSGER)	18.70 (18.22)	0.61 (-2.06)	D (ND)	+2.67	.03
	SB (NOSGER)	19.78 (19.00)	0.83 (-0.56)	ND (ND)	+1.39	NS
	IADL/SB	38.48 (37.22)	1.43 (-2.61)	ND (ND)	+4.04	.03

Table 1 *(continued)*

first author, year	outcome measures - functional	baseline intervention (control)	Intervention (control) change from baseline	p value	net change between groups	p value
Dysken, 2014	ADCS-ADL	57.20 (56.93	-13.81 (-16.96)	ND (ND)	+3.15	.03
	Dependence Scale	-	-	ND (ND)	-	NS
	CAS	2.7 (3.0)#	3.35 (5.14)	ND (ND)	-1.79	NS
Foster, 1996	CGIC	-	-	NS (NS)	-	NS
	ADAS-noncog	5.3 (4.8)	-0.7 (-0.3)	NS (NS)	-0.4	NS
Freund-Levi, 2006	CDR	1.0 (1.1)	0.1 (0.0)	NS (NS)	0.1	NS
	CDR sum of boxes	5.8 (6.0)	0.4 (0.5)	NS (NS)	-0.1	NS
Shah, 2013	ADCS-ADL	57.95 (57.38)	-3.74 (-3.66)	ND (ND)	-0.08	NS
	CDR sum of boxes	6.18 (6.45)	0.77 (0.69)	ND (ND)	+0.08	NS
Sun, 2007	simplified Barthel ADL Index	ND (ND)	-0.33 (-0.19)	ND (ND)	-0.14	NS

$ mild: MMSE 21-26, moderate: MMSE 10-20, severe: MMSE <10, according to NICE TA 217 [30] except for Arlt 2012 and Dysken 2014, where author's description is given (no MMSE range was specified) * only figures of results available, no exact numbers ; # median. AD, Alzheimer's Disease; ADAS-cog, Alzheimer's Disease Assessment Scale, cognitive subdomain; ADAS-noncog, Alzheimer's Disease Assessment Scale, non-cognitive subdomain; ADCS-ADL, Alzheimer's Diseae Co-operative Study Activities of Daily Living; BADL, Basic Activities of Daily Living; CAS, Caregiver Activity Survey; CASI, Cognitive Abilities Screening Instrument; CDR, Clinical Dementia Rating scale; CGIC, Clinical Global Impression of Change; DSST, Digit Symbol Substitution Test;; IADL, Instrumental Activities of Daily Living; MMSE, Mini Mental State Exam; ND, not done; NOSGER, Nurses Observation Scale for Geriatric patients; NS, non-significant; SB, Social Behavior; TMT, Trail Making Test; UK, unknown

Oxidative stress

Three studies[21,22,28] examined the added effect of antioxidants to cholinesterase inhibitor therapy. Arlt et al.[21] found that vitamin C and E in addition to donepezil had a slightly negative effect on cognition (MMSE score decreased compared to baseline in the vitamin group). Cornelli et al.[28] found that Formula F had no or minimal effect on cognition (more cases improved on MMSE II score in treatment arm compared to placebo arm). Dysken et al.[22] found no effect of vitamin E in addition to a cholinesterase inhibitor on cognition but did show a reduction in the rate of functional decline over a mean follow-up of 2.27 years. The other two studies did not assess functional outcome. Taking into account the low to moderate risk of bias, there is little evidence of a benefit of anti-oxidants in combination with cholinesterase therapy on cognition. There is, however, support for decreased rate of functional decline in mild to moderate male patients with AD taking vitamin E in addition to cholinesterase inhibitors.

Acetylcholine precursors

Two studies[23,24] reported the added effect of acetylcholine precursors to cholinesterase therapy. As found by Amenta et al.[24], choline alphoscerate in addition to donepezil had a modest positive effect on cognition and function, relative to the intervention group, the placebo group showed a decline in cognition after 12 months follow-up. In contrast, Foster et al.[23] found no effect of lecithin in addition to tacrine on cognition and function. With a moderate risk of bias for Amenta et al.[24] and a low risk of bias for Foster et al.[23], the data do not suggest a positive effect of acetylcholine precursors in combination with cholinesterase inhibitors on cognition or function.

Increased homocysteine levels

Four studies[25,27-29] reported nutritional interventions that are related to reducing high homocysteine levels. Connelly et al.[27] reported a modest positive effect of folic acid in addition to a cholinesterase inhibitor on cognition and a modest positive effect on functional outcome. The number of 'responders' according to NICE criteria was higher in the intervention group receiving both a cholinesterase inhibitor and folic acid in contrast to the comparison group which only received a cholinesterase inhibitor. A significant difference in change from baseline in combined Instrumental Activities of Daily Living and Social Behaviour score was observed between the groups. Two studies[25,29] observed no differences in cognition or function between treatment with only conventional AD medication (cholinesterase inhibitor with or without memantine) and the combined treatment with AD medication and B vitamin (B6, B12 and folic acid) containing supplements . Cornelli et al.[28] found that Formula F, containing among other nutrients vitamin B6, B12 and folic acid, had a slight effect on cognition (more cases improved on MMSE II score in treatment arm compared to placebo arm). This study did not assess functional outcome. All studies had a low risk of bias. Thus, whereas a positive effect of folic acid in combination with cholinesterase inhibitors on cognition and function was observed in the study by Connelly et al, such was not the case, or only minimal, for the other studies, which investigated the combination of folic acid with other B vitamins and additional nutrients.

Membrane fluidity and inflammation

Two studies reported on effects of omega-3 fatty acids[26,31] or omega-3 fatty acids containing supplements[29] in conjunction with cholinesterase inhibitor therapy. In both studies, the addition of omega-3 fatty acids (DHA and EPA) to a cholinesterase inhibitor had no effect on function and minimal or no effect on cognition compared to treatment with only a cholinesterase inhibitor. Modest improvements were only observed by Freund-Levi et al.[26] in very mild cases of AD. Given the low risk of bias of both studies the current evidence does not support a positive effect of omega-3 fatty acids in combination with cholinesterase inhibitors on cognition or function.

Discussion

In this review, we found little evidence that adding antioxidants, acetylcholine precursors, B-vitamins or omega-3 fatty acids to cholinesterase inhibitor therapy has a positive effect on cognition or activities of daily life in patients with Alzheimer's disease. The use of vitamin E supplements could reduce the rate of functional decline when combined with cholinesterase inhibitors in only one study, whereas cognition was not affected in both this and other studies. Interestingly, although beyond the scope of this review, the beneficial effect of vitamin E was diminished by adding memantine. Regarding B-vitamins one study observed a beneficial effect with folic acid[27], whereas other RCTs comprised of patients with more advanced AD found that adding B-vitamins including folic acid to a cholinesterase inhibitor had no effect on cognition or function[25,29]. Recent systematic reviews focusing on single nutrients or nutrient groups only, have also failed to find an effect on cognition in cognitively impaired and/or unimpaired people[16,32,33].

The diversity of nutritional supplements investigated in the included studies is large, which exemplifies the quest for nutritional intervention but impedes direct comparisons. However, the heterogeneity between studies allows for the opportunity to examine multiple disease pathways and compare studies, grouped by their disease modifying factors. The heterogeneity of the included studies provides a broad overview of the topic, and therefore, our conclusions are not limited to one domain.

Although the evidence did not support the effect of nutritional supplements on cognition or function, several studies did find a positive effect on intermediate outcomes, suggesting effects on the underlying mechanism they were trying to influence[21,25,28]. For instance, Arlt et al.[21] found oxidation of cerebral spinal fluid (CSF) lipids to be reduced after one year of supplementation with the antioxidants vitamin C and E, while the clinical course of AD over one year was not affected.

There are three possible explanations for the absence of an effect on cognition and function, in the presence of a biological effect. Firstly, administration of supplements may be too late for patients

who have already developed overt AD. In these patients, although the underlying causative mechanism can be positively influenced, the damage that has been caused in the brain resulted in cognitive impairment, and is thus too large to be restored. Trials in patients with (very) mild cognitive impairment, or at high risk to develop cognitive decline, would help clarify this issue, provided that the follow-up time is sufficient to monitor progression to dementia. Unfortunately this is often not the case, as in Lee et al.[34], who reported improvement in cognition in MCI patients after 12 months DHA treatment but provided no data on conversion to AD. In such cases, a 3 year follow-up period would likely be required to adequately assess disease progression. However, Petersen et al.[35] could not identify an effect of 3 year treatment with vitamin E on progression to AD. Thus, intervention may be necessary even before the onset of mild cognitive problems. Trials in large numbers of cognitively healthy people over long periods of time may however not be feasible, unless they contain an enriched population with multiple risk factors.

Second, even more than two disease-modifying mechanisms may have to be addressed, in order to alter cognition and functional outcome. Multi-nutrient formulas have been developed for this purpose, such as formula F™ and Souvenaid™ (both included in this review). These have yielded mixed results and some controversy in literature but there may be larger positive effects, restricted to mild AD cases[28,36,37]. Combining physical and cognitive training with nutritional interventions have been explored as well, again with mixed results[38,39]. Altogether, the available research has not yet provided convincing evidence that combining multiple treatments is more beneficial in AD than a single treatment. More research is needed on which nutritional supplements and other treatments should be combined, as well as on which study designs are most appropriate for this. Currently, quality indicators have been developed for studies reporting on combined interventions, underlying the specific methodological issues in such complex interventions[40,41]. Ideally studies investigating the combined use of two treatments apply a factorial design, sufficiently powered to detect the interaction effects, and mostly including four groups: a true control group without intervention, two groups receiving either treatment alone and a group receiving both treatments. This can of course be extended to multiple combinations. In addition, an optimal treatment duration and follow-up time is required to monitor the difference in disease progression. A scientifically sound example is the factorial design used by Dysken et al.[22], where a follow-up of up to 4 years allowed for modeling the rate of decline. Future studies that take above recommendations into account can advance our knowledge and move the field to a higher level.

Last, multiple trajectories may result in Alzheimer's disease, making the disease not only multifactorial but adding to phenotypical heterogeneity as well. Different modifiable factors may underlie disease progression in different patients, and therefore personalized interventions may be necessary to address these specific needs. For instance, in one patient high homocysteine levels and inflammation may be more prominent, while for another patient oxidative stress and decreased membrane fluidity may be key factors. Some studies provide evidence for this

paradigm. For instance, Lloret and colleagues[42] investigated vitamin E supplements and oxidative stress in patients with AD. For those patients in whom vitamin E supplementation led to lower oxidative stress (lower blood oxidized glutathione levels), cognitive function was maintained. In contrast, among patients whose oxidative stress was not reduced by treatment with vitamin E, an accelerated cognitive decline was observed. This supports the idea that personalized medicine can lead to better treatment effects, especially in Alzheimer's disease which may be multifactorial in etiology and has proven to be heterogenic in its presentation[18,43].

Limitations

Because unpublished and grey literature (informally published written material) were not included in this review, publication bias may have affected the presented results. However, a funnel plot analysis could not be performed due to insufficient amount of data and the heterogeneity between outcome measures. Several studies with negative results were identified nonetheless, implying minimal publication bias. Since this review was restricted to English language publication, potentially eligible studies in other languages may have been excluded from this review.

Conclusion

In this review multiple nutritional interventions in addition to cholinesterase inhibitor therapy were examined. Only the use of vitamin E supplements could reduce the rate of functional decline when combined with cholinesterase inhibitors in one study, whereas cognition was not affected in both this and other studies. None of the other nutritional supplements showed convincing evidence of a significant effect on cognition or daily functioning. However, cognitive and functional improvement is difficult to achieve in patients with AD, despite epidemiological evidence and biological effects of nutritional supplements. This is most likely due to the multifactorial nature of AD, apart from effects of right timing of the interventions, and appropriate trial design and duration. Thus, addressing one pathway in addition to cholinesterase inhibitor therapy is likely insufficient to influence symptoms, let alone alter the course of the disease. A personalized and multifactorial intervention may potentially have a greater impact on symptoms and clinical signs from the earliest course of the disease onwards. A more personalized approach to treatment in early AD should be investigated as only this treatment type is tailored to the individual patient's (multidomain) deficiencies and disease mechanisms. In combination with early diagnosis and intervention , personalized medicine will be necessary to combat Alzheimer's disease.

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Appendix A

Pubmed search strategy:

Search strategy:	created by LL
Date search:	16-01-2012
Limits:	None
Hits:	367
Initial selection:	by LL and OM

Search strategy:

1. "Dementia"[Mesh]
2. dementia*[tiab]
3. "cognitive impairment"[tiab]
4. "cognitive function"[tiab]
5. "cognitive performance"[tiab]
6. "cognitive decline"[tiab]
7. "cognitive loss"[tiab]
8. "cognitive dysfunction"[tiab]
9. "cognition disorders"[MeSH Terms]
10. "cognition disorders"[tiab]
11. "cognitive disorders"[tiab]
12. "alzheimer disease"[MeSH Terms]
13. alzheimer*[tiab]
14. "mild cognitive impairment"[tiab]
15. MCI[tiab]
16. "Dementia, Vascular"[Mesh]
17. "vascular dementia"[tiab]
18. OR/1-17
19. "factorial design"[tiab]
20. factorial[tiab]
21. "Randomized Controlled Trials as Topic"[Mesh]
22. "Randomized Controlled Trial"[Publication Type]
23. "random allocation"[Mesh]
24. "double blind method"[Mesh]
25. RCT[tiab]
26. "clinical trial"[tiab]
27. random*[tiab]
28. controlled[tiab]
29. "double blind"[tiab]
30. "single blind"[tiab]
31. "multidomain intervention"[tiab]
32. "multidisciplinary intervention"[tiab]
33. "multicomponent intervention"[tiab]
34. OR/19-33
35. interaction*[tiab]
36. "complex intervention*" [tiab]
37. "combination therapy"[tiab]
38. "combination treatment"[tiab]
39. multicomponent[tiab]
40. "Exercise"[Mesh]
41. "Movement"[Mesh:noexp]
42. "Physical therapy modalities"[Mesh]
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46. "life style"[tiab]
47. lifestyle[tiab]
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51. "Cholinesterase Inhibitors"[Mesh]
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53. "nmda receptor antagonist"[tiab]
54. "nmda antagonist"[tiab]
55. galantamine[tiab]
56. rivastigmine[tiab]
57. donepezil[tiab]

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68. food[tiab]
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71. supplement*[tiab]
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74. "dietary supplement"[tiab]
75. homocystein[tiab]
76. antioxidant*[tiab]
77 vitamin*[tiab]
78. "omega 3"[tiab]
79. "folic acid"[tiab]
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83. tocopherol[tiab]
84. selenium[tiab]
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86. PUFA[tiab]
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89. "omega 6"[tiab]
90. "n-6"[tiab]
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111. OR/109-110
112. 18 AND 34 AND 59 AND 108 NOT 111

Appendix B

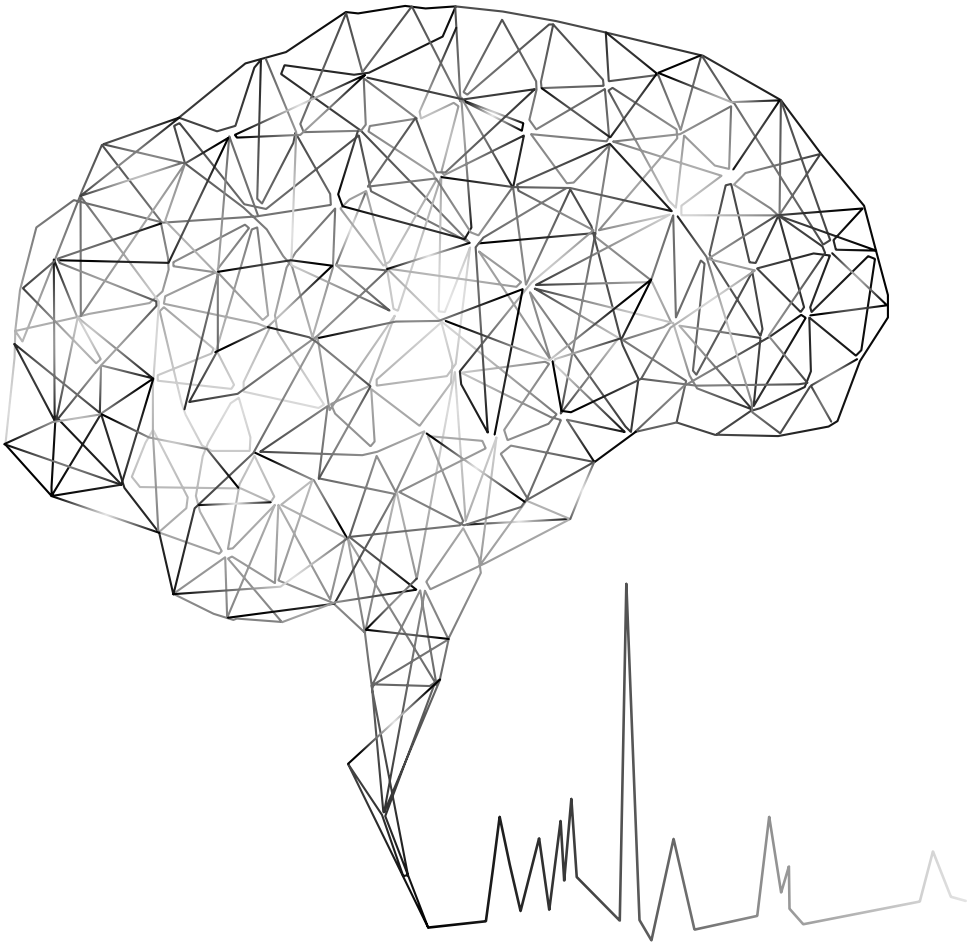
The Cochrane Library search strategy:

Search strategy:	created by LL
Date search:	24-01-2012
Limits:	None
Hits (Trials):	112
Initial selection:	by LL and OM

Search strategy:

dementia
cognitive impairment
1 OR 2
vitamin
antioxidant
nutrition
4 OR 5 OR 6
3 AND 7

6
Summary



Key findings

- Circulating levels of nutrients, relevant for the phospholipid synthesis pathway and known to be decreased in the AD population, can be increased within weeks in patients with mild AD by a multi-nutrient intervention.

- Uptake of nutrients reaches a plateau phase for most nutrients during prolonged intake, thus increasing the availability of precursors and cofactors in the circulation that may be used for the formation and function of neuronal membranes and synapses in the brain.

- Increased levels of phosphocreatine in regions that show early degeneration and an increased tissue pH suggest that brain energy metabolism is altered in mild AD.

- Although preclinical and post-mortem studies have shown alterations in membrane function and lipid composition in AD, no alterations in phospholipid metabolism were found *in vivo* in mild AD.

- Short term multi-nutrient supplementation affects brain phospholipid metabolism in patients with mild AD.

- Short term multi-nutrient supplementation does not alter neural integrity in patients with mild AD.

- The addition of vitamin E to cholinesterase inhibitor therapy in AD may slow functional decline.

- The addition of nutritional supplements to cholinesterase inhibitor therapy in AD does not reduce cognitive decline.

AD is a progressive neurodegenerative disease affecting more than 20 million people worldwide and is the leading cause of dementia in older persons. Memory loss, and problems with planning, language and wayfinding are among its devastating effects. Despite immense research efforts, a cure for AD is not available. Several lifestyle factors have been identified that can reduce or increase the risk for dementia or AD, such as diet. This has raised much interest in the role nutrition plays in AD, and whether nutrients or nutritional supplements can affect the course and the pathology of this disease. One of the pathological pathways that may be amenable to nutritional intervention, is the brain's phospholipid metabolic pathway. Any drug or nutritional intervention aiming to prevent, reduce, or reverse cognitive symptoms in AD must find its way to the circulation and onwards to the brain in order to affect clinical outcome. The research in this thesis explores the effects of nutrients in AD, with a focus on those nutrients that are involved in phospholipid metabolism, at several levels of this route.

In chapter 2 we investigated the first step in the route from nutrient intake to cognitive effects. Data from three RCTs representing more than 900 people with mild to moderate AD and including both drug-naïve patients as well as patients on AD medication were analyzed. We showed that a multi-nutrient intervention can increase plasma and/or erythrocyte micronutrient levels (uridine, choline, selenium, folate, DHA, EPA, and vitamins B6, B12 and E) after 12-24 weeks. Uptake of most nutrients was observed already after 6 weeks and reached a plateau between 24 and 48 weeks of daily intake. Analyses on a subset of mild AD patients revealed no effects of this multi-nutrient combination on markers of inflammation or oxidative stress. To conclude, circulating levels of micronutrients can be reliably increased in a diverse AD population by a multi-nutrient intervention. This forms the basis for any effects these nutrients can have on phospholipid metabolism, neuronal membrane formation, and ultimately synaptic function and cognition.

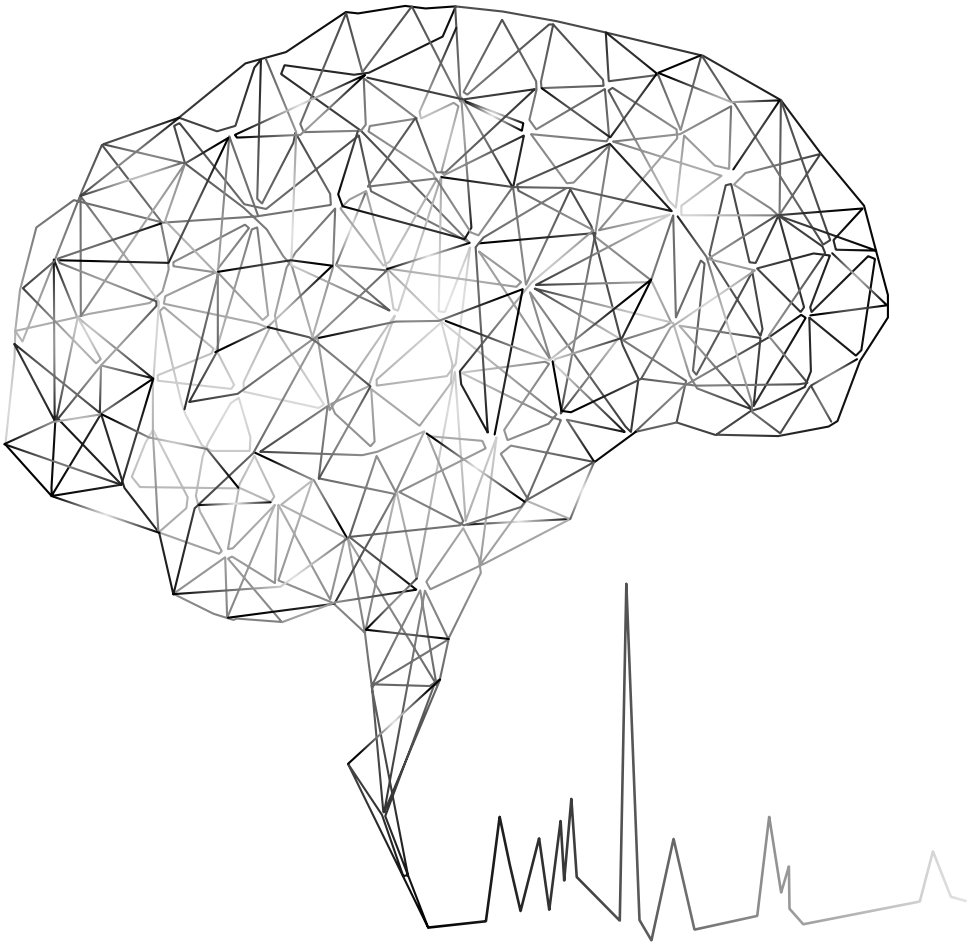
In the next chapter we examined brain phospholipid and energy metabolism *in vivo*, using state-of-the-art techniques, in a large group of patients with AD and healthy older adults. Contrary to our expectations, and despite various reports of post mortem phospholipid membrane alterations, we did not observe any differences in phospholipid metabolism in patients with mild AD compared to their age-matched controls. However, we did see alterations in energy metabolism in AD. Most interestingly, phosphocreatine, an important energy molecule, was increased in those regions that show early degeneration in AD, but not in a region that is involved at a later stage.

Although phospholipid metabolite levels were not different from normal, we showed in chapter 4 in a double-blind RCT that it is possible to influence brain phospholipid metabolism through a multi-nutrient intervention. After 4 weeks, the ratio of phospholipid precursors (i.e. phosphomonoesters) to phospholipid breakdown products (i.e. phosphodiesters) was significantly increased in mild AD patients that received this investigational product once daily compared to patients that received a control product. This shows that the nutrients, or their

metabolites, cross the blood brain barrier and affect the metabolic pathway for which they are substrates. However, measures of neural integrity were unaffected by this intervention.

Finally, in chapter 5 we turned to other multi-domain interventions and their effect on cognition and daily function. Specifically, in a systematic review we looked at single and multi-nutrient supplements as an addition to cholinesterase inhibitor therapy. Only for vitamin E some evidence was found to support that addition to cholinesterase inhibitor therapy may slow functional decline. However, cognitive decline was not affected by addition of vitamin E nor any other nutritional supplement. This shows that cognitive and functional improvement is difficult to achieve in patients with AD, despite epidemiological evidence of risk reduction and biological evidence of the effects of nutritional supplements.

7
Discussion



^{31}P-MRSI uncovered anatomical pattern of energy abnormality in AD

In this thesis we describe the largest study to date that investigated key molecules of energy and phospholipid metabolism in AD by ^{31}P-MRS (chapter 3). Recent advances in this technique allowed us to perform whole brain measurements in under 15 minutes. This enabled us to assess high-energy phosphates, phospholipid metabolites and pH in four brain regions of interest in AD simultaneously. At the start of this study we expected to mainly find differences between AD patients and healthy controls in phospholipid metabolites. However, we did not observe any differences in phospholipid metabolism. Instead, a novel finding emerged from this investigation. We found for the first time increases in PCr, PCr/Pi and pH in mild AD patients compared to age- and gender-matched controls, indicating an altered energy metabolism in AD. In order to understand the relevance of this finding, we need to briefly explore energy metabolism in the brain.

High-energy phosphates play a central role in energy metabolism in cells, forming the currency of energy production and utilization[1]. ATP is necessary for all cellular processes that require energy, whether it is the maintenance of the membrane potential or neurotransmitter release from a presynaptic site. PCr functions as a temporal and spatial energy buffer to quickly replenish ATP when energy demands suddenly or locally increase in tissues with a high and fluctuating energy consumption. It also functions as a buffer to enhance the efficiency of mitochondrial oxidative phosphorylation by keeping ADP levels available in sufficient amounts. In the brain, two isoforms of the enzyme creatine kinase (CK) are present, cytosolic brain type CK (BCK) and ubiquitous mitochondrial CK (uMtCK), that convert PCr, ADP and one proton (H^+) to ATP and creatine, or vice versa. Usually, ATP is then immediately utilized to release its energy potential, and ADP and Pi are generated. In muscle this is clearly seen when upon exercise PCr concentrations drop and Pi levels rise, with ATP levels remaining stable. In brain tissue, the situation is more complex as the brain is always active and fluctuations in energy demand are more local and likely faster.

In the study described in chapter 3 of this thesis we looked at steady state levels of PCr, ATP and Pi and at intracellular pH in AD and normal aging. Because we acquired whole brain data, we could determine that the increase in PCr in patients with AD was not uniform across the four investigated brain regions. Increased levels were found in the RSC and both hippocampi, regions that are known to be involved early on in the disease, whereas levels in the ACC, a region affected later in the course of AD, were unaltered. This resembles the anatomical pattern seen in tau pathology, glucose hypometabolism and atrophy. We can now add these new findings to the biomarker graph from the introduction in Figure 1. The *in vivo* alterations we found in our research in mild AD patients corroborate post-mortem findings of impaired CK enzyme activity and indicate that changes in energy metabolism are not just present in end-stage disease. Together with the distinct anatomical pattern of PCr abnormalities, this suggest that these processes may be important in the pathophysiology of AD.

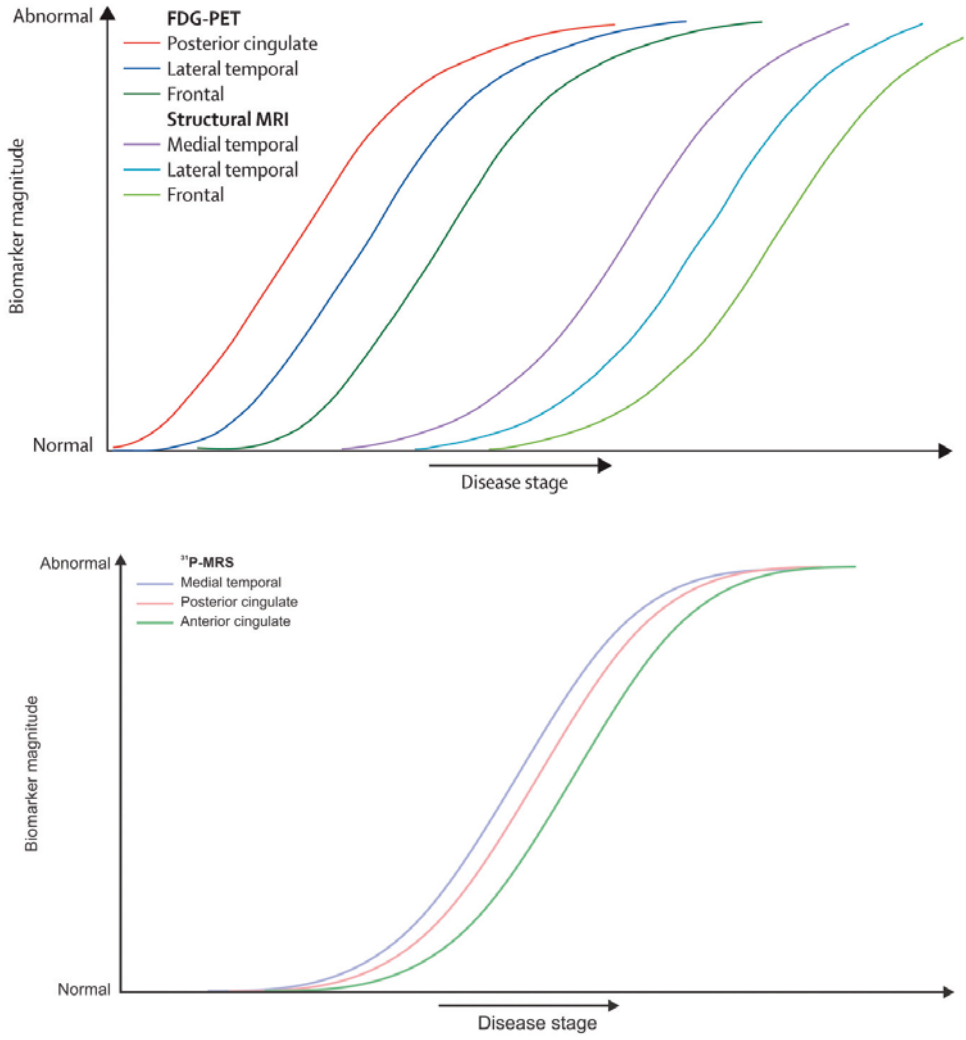


Figure 1

Hypothetical graph of appearance of imaging biomarker abnormalities in different brain regions, updated from Figure 1 in the introduction. Top panel, Anatomical variation exists in the time courses of biomarker abnormalities within imaging modes (FDG-PET and structural MRI). Bottom panel, similarly to FDG-PET and structural MRI, one would expect abnormalities in phosphocreatine to appear in the following order based on the current results: medial temporal lobe, retrosplenial cortex/posterior cingulate, anterior cingulate. Top panel reprinted with permission from Jack et al. (2010)[2].

In future research, the first step would be a whole brain analysis (of the current dataset) to expand our description of the anatomical pattern of PCr increase. In addition, magnetization transfer experiments to determine the CK and ATPase reaction rates would extend the current steady-state findings. Finally, the investigation of at-risk populations as well as correlation of energy metabolites with other pathologies or with cognitive performance would be particularly interesting to determine the time course and significance of these alterations in energy metabolism.

Phospholipid metabolism alterations may be a late stage process in AD

One of the most surprising findings from the work in this thesis, or actually lack of a finding, is the absence of differences in phospholipid metabolites between patients with AD and healthy aged controls. This raises the question whether phospholipid metabolism and phospholipid content of neuronal membrane are pathologically relevant to AD. Previous studies that used post-mortem brain tissue showed that the brain's major phospholipids PE and PC were decreased in AD in several regions[3]. Additionally, phospholipid catabolic enzyme activity is decreased and activity of phospholipid re-synthesis enzymes is increased compared to controls[4]. Furthermore, *ex vivo* biochemical analyses have detected that the DHA content of neuronal membrane was decreased[5,6], thereby influencing membrane fluidity. This is expected to have a direct influence on cell signaling pathways because membrane fluidity affects synaptic vesicle release, receptor recycling and second messenger signaling[7,8]. This is all compelling evidence that phospholipid metabolism and phospholipid membrane composition is altered in AD. So why did we not observe any differences *in vivo* between AD patients and healthy aged controls in our study? The conclusion may have to be that phospholipid metabolism is not yet affected at this stage. Any post mortem analysis is most likely from end-stage AD, while the patients in our study were recently diagnosed, and classified as mild AD. Although we must consider the possibility that our measurements lacked the sensitivity or specificity to detect differences in the levels of MRS observable phospholipid metabolites, this is not very plausible as we could demonstrate changes in these metabolites after a short nutritional intervention. Thus, most likely, abnormalities in phospholipid metabolism take place at the later stages of AD.

To further examine the time course of phospholipid alterations in AD, it would be necessary to repeat these measurements at several stages of the disease. A longitudinal study would be preferable, but a cohort study with pre-clinical, mild and severe AD patients could be a first step. This would allow the detection of changes in metabolite levels over the course of the disease. We should be aware though that changes in biomarkers over time are often not linear and that the precise pattern may only be discovered using longitudinal studies.

Brain phospholipid metabolism can be influenced by a short term multi-nutrient intervention

If we are still confident that phospholipid metabolism is pathologically relevant to AD, does it make sense to support phospholipid formation before deficits develop? Actually, this is exactly

the direction in which the field is heading. Early intervention is likely of essence, should we have any change of fighting neurodegenerative diseases[9].

In chapters 2 and 4, we demonstrated that plasma and erythrocyte levels of nutrients, that have a role in the phospholipid synthesis pathway, can be reliably increased after only 4 weeks in patients with mild AD and that most levels reach a plateau phase after sustained intake between 24 and 48 weeks. Moreover, in chapter 4 we showed that short term daily supplementation with these same nutrients increases the ratio of phosphomonoesters to phosphodiesters. Thus, the balance between building blocks and breakdown products of phospholipids is altered, which indicates that intake of these nutrients affects phospholipid metabolism. As neither phosphomonoester signals nor phosphodiester signals were significantly changed after 4 weeks, we were unable to determine whether this is due to increased phospholipid formation or to decreased phospholipid breakdown. Our results did indicate that the increase is driven by changes in the ethanolamine-containing phospholipids and that the effect on choline-containing phospholipids is smaller or absent. This is not to say that brain choline levels cannot be increased, as the total level of choline-containing compounds (mainly choline-containing phospholipids and free choline) was higher after 4 weeks of the intervention compared with intake of a control product. A slight difference in ^{1}H and ^{31}P-MRS brain volumes could be underlying this discrepancy between the results on choline-containing phospholipids from the different MRS measurements.

Besides their effect on the phospholipid synthesis pathway, we also expected this multi-nutrient intervention to slow down changes in brain metabolites related to neural integrity and gliosis. For instance, DHA has anti-inflammatory effects[10] (which may be relevant for gliosis) and both uridine and DHA can influence neurite outgrowth[11,12] (which could result in improved neuronal health). However, this short term intervention did not affect brain levels of NAA, a marker for neural integrity, levels of mI, a marker for gliosis, or the ratio NAA/mI. Possibly, the normal rate of change of these metabolites over 4 weeks time is limited, such that a deceleration effect of the intervention is not yet discernible, or it may be that the damage that these metabolites reflect is already irreversible at this stage of the disease.

Our results show that we *can* influence those processes that are not yet affected in mild stage AD (i.e. phospholipid metabolism), but not those that are already disturbed at this stage (neural integrity and gliosis). This suggests that a higher gain may be expected with earlier intervention, when pathology is less advanced. The field has been moving towards earlier interventions in recent years[9] and several trials are underway that even try to prevent cognitive decline in those genetically predisposed to develop AD[13,14]. The multi-nutrient intervention that was the focus of this research, is investigated in an ongoing trial in a group of individuals at risk for developing AD (i.e. having mild cognitive impairments [MCI]), with the aim to reduce or prevent cognitive decline. Those results will shed more light on whether earlier intervention is indeed associated with a higher gain, and whether intervention at this MCI-stage is early enough.

Nutritional interventions in AD: too little, too late, and too general

Although the majority of the research in this thesis concerns the biology of AD and the biological effects of nutrients, in chapter 5 we turned our attention to the clinical endpoints in AD. In a systematic review, we investigated what happens with cognition and daily function when a nutritional supplement is added to cholinesterase inhibitors, one of the two drugs with proven, albeit modest and temporary, efficacy in AD. This yielded rather disappointing results, as no supplement could improve cognition or slow cognitive decline and only slim evidence was provided for improved daily function with the addition of vitamin E. Despite mounting epidemiological evidence on the role of nutrition in cognition and dementia[15,16], interventions with nutritional supplements repeatedly fail to show an effect on cognition. This is not only the case for people that already have AD[17-19], but also in cognitively healthy (elderly) populations the prevention of cognitive decline or dementia is difficult to achieve[20]. Interestingly, many interventions do show biological effects on the pathways they aim to influence. For instance, B-vitamins do lower homocysteine levels in hyperhomocysteinemic cognitively intact older persons but do not improve cognitive performance[21], and anti-oxidant supplements reduce markers of oxidative stress in cerebrospinal fluid (CSF) in AD patients, but without clinical efficacy[22]. This biological efficacy without clinical benefit alludes to three facts that may influence the current lack of successful trials to reduce or prevent cognitive decline. Firstly, interventions may be initiated too late. The commonly held view nowadays is that neurodegenerative diseases like AD start decades before the onset of symptoms[9]. Even in the aged population, pathological processes may be too advanced for interventions to be able to counteract or repair the damage that causes cognitive decline. Secondly, a multifactorial disease like AD may require simultaneous intervention on multiple domains. Multiple causes of AD may have to be addressed in order to improve clinical outcome. A recent trial showed modest success when diet, vascular health, and social health were all addressed together in a population of community-dwelling elderly[23]. Finally, AD is a highly heterogenic disease. Therefore, clinical effects of interventions may be lost on a group level when the dominant biological pathways that contribute to cognitive impairment are different for each individual. A recent trial aimed at reducing cardiovascular risk factors in community-dwelling older persons failed to reduce the incidence of dementia and cardiovascular disease after 6 years[24]. This lack of efficacy may be partly ascribed to a high standard of usual care, but one could also speculate that the intervention effect was diluted by individuals in which cardiovascular health did not contribute to cognitive impairment. To tackle all three issues, we should aim for early multi-domain interventions that are tailored to the individual patient.

Clinical implications and future perspectives

Personalized medicine in nutritional interventions

As discussed above, the pathway to AD may be different for each individual. In each individual a different combination of several pathological processes leads to cognitive impairment. This is illustrated in Figure 2, where hypothetical graphs are shown for three different individuals. In patient 1, five factors equally contribute to his impairment. The cognitive impairment of patient 2 is much more dominated by amyloid beta and tau pathologies. In a third patient, oxidative stress and membrane dysfunction are the dominant pathologies. One can imagine that reducing ROS by anti-oxidants does not do much for patient 2, while it may be of benefit to patient 3. But the largest efficacy may be expected in patient 3, when both oxidative stress and membrane dysfunction are addressed. Of course, this requires knowledge of the pathways that are relevant at an individual level.

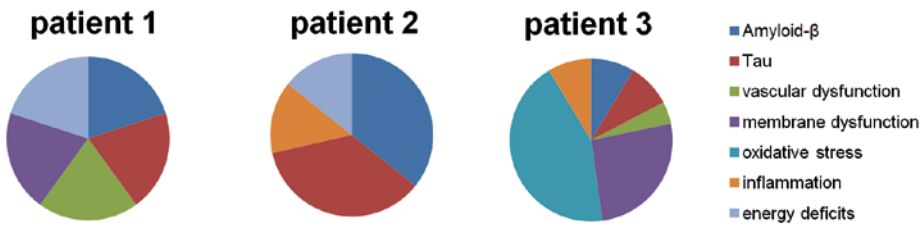


Figure 2

Hypothetical graphs of the contribution of pathological pathways to cognitive dysfunction in different patients.

The current era of personalized medicine places emphasis on tailored healthcare in contrast to a 'one size fits all' approach. The European Alliance for Personalised Medicine defines personalized medicine as 'a targeted approach to the prevention, diagnosis and treatment of disease based on an individual's specific profile[25]. This can also be applied to nutritional interventions as they can be tailored to different underlying pathological mechanisms and to individual deficiencies and heightened needs. However, many factors besides the efficacy of such interventions influence their prospects. Time and costs of diagnostic tools, costs of making individually tailored supplements or personalized diet advice, lack of user convenience, and lack of resources may be obstacles for implementation. Notwithstanding, the anticipated gain is a great motivator to tackle the obstacles we may be faced with, increasing the odds for successful implementation.

^{31}P-MRS based biomarkers

In our research we used state-of-the-art MR spectroscopic techniques that uncovered abnormalities in energy metabolism in early stage AD with a distinct anatomical pattern. We speculate that this anatomical pattern reflects sequential involvement of brain areas, as has been shown for other pathologies in AD (Figure 1). At the same time, our results suggest that abnormalities in phospholipid metabolism may become relevant only at a late stage in the disease. In Figure 3 we have added our findings to the hypothetical model of AD biomarkers and their relationship with clinical disease stage[9]. Next to the current neuroimaging techniques PET, fMRI and volumetric MRI, that provide knowledge on amyloid beta accumulation, synaptic dysfunction and brain structure, we can now add ^{31}P-MRSI that gives us information on energy and phospholipid metabolism. The question comes to mind what role ^{31}P -MRSI could have in monitoring disease progression and whether ^{31}P -MRS based biomarkers would be feasible.

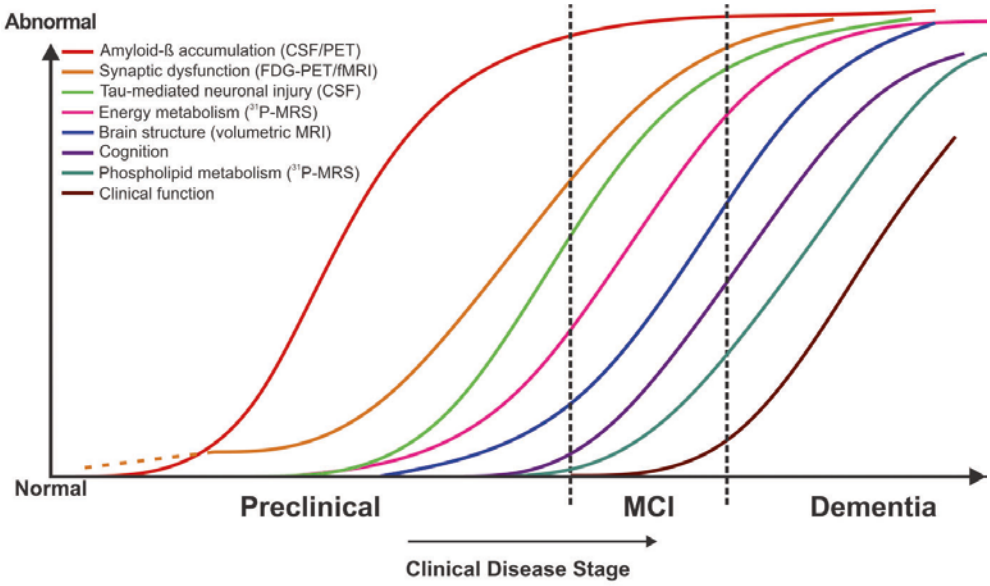


Figure 3

Hypothetical model of dynamic biomarkers. Aβ as identified by cerebrospinal fluid Aβ42 assay or positron emission tomography (PET) amyloid imaging. Synaptic dysfunction evidenced by fluorodeoxyglucose PET (FDG-PET) or functional magnetic resonance imaging (fMRI), with a dashed line to indicate that synaptic dysfunction may be detectable in carriers of the 3/4 allele of the apolipoprotein E gene before detectable Aβ deposition. Neuronal injury is evidenced by cerebrospinal fluid tau or phospho-tau, brain structure is evidenced by structural MRI. Energy and phospholipid metabolism abnormalities are evidenced by phosphorus MR spectroscopy (^{31}P-MRS). Biomarkers change from normal to maximally abnormal (y-axis) as a function of disease stage (x-axis). The temporal trajectory of two key indicators used to stage the disease clinically, cognitive and behavioral measures, and clinical function are also illustrated. Adapted with permission from Sperling et al. (2011)[9].

It is certain that this technique adds a unique insight in disease processes that cannot be measured *in vivo* in any other way. Moreover, like conventional MRI, it is non-invasive and low risk compared to FDG-PET and CSF measures, making it especially suitable for repeated measurements. However, there are also barriers for implementation. Compared to proton MRI, phosphorus has a low intrinsic signal intensity, hindering measurements at a high resolution within a (clinically) feasible timeframe. In turn, low resolution increases partial volume effects that will contribute to between-subject variability. Practically, current clinical implementation of ^{31}P-MRS is hindered by the fact that most clinical MR systems are not normally equipped for nuclei other than proton (i.e. X-nuclei, such as ^{31}P). However, in light of upcoming developments in clinical MR, great progress is expected from 7 tesla MR systems[26], which will allow for ^{31}P -MRSI at a higher spatial resolution. Additionally, with the current findings of PCr abnormalities with anatomical variation in AD, it would be particularly interesting to explore the feasibility and relevance of PCr imaging. Since PCr is the dominant peak in the cerebral ^{31}P-MR spectrum, measurement time can be reduced if the purpose would only be to reliably measure PCr, compared to when an entire spectrum needs to be (reliably) obtained. Hereby, whole brain PCr imaging with a reasonable resolution and within a clinically acceptable timeframe becomes attainable. If PCr abnormalities prove to be specific for AD, these measurements would bring the use of PCr as a biomarker of AD within reach.

Concluding remarks

The research in this thesis explored the effects of nutrients in AD, with a focus on those nutrients that are involved in phospholipid metabolism. We demonstrated that both circulating levels as well as essential brain metabolic processes can be influenced by a multi-nutrient supplementation that contains precursors and cofactors for the synthesis of membrane phospholipids. Ultimately, this may reduce cognitive decline by supporting synapse function. However, at the moment, there is no evidence that the addition of nutritional supplements to standard AD medication reduces cognitive decline in mild AD patients. The current research also led to the discovery of abnormalities in high-energy phosphate metabolism in mild AD with a distinct anatomical pattern. With future technical advances in (phosphorus) MR, phosphocreatine may become a novel MR-based biomarker of AD.

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8

Nederlandse samenvatting | List of abbreviations
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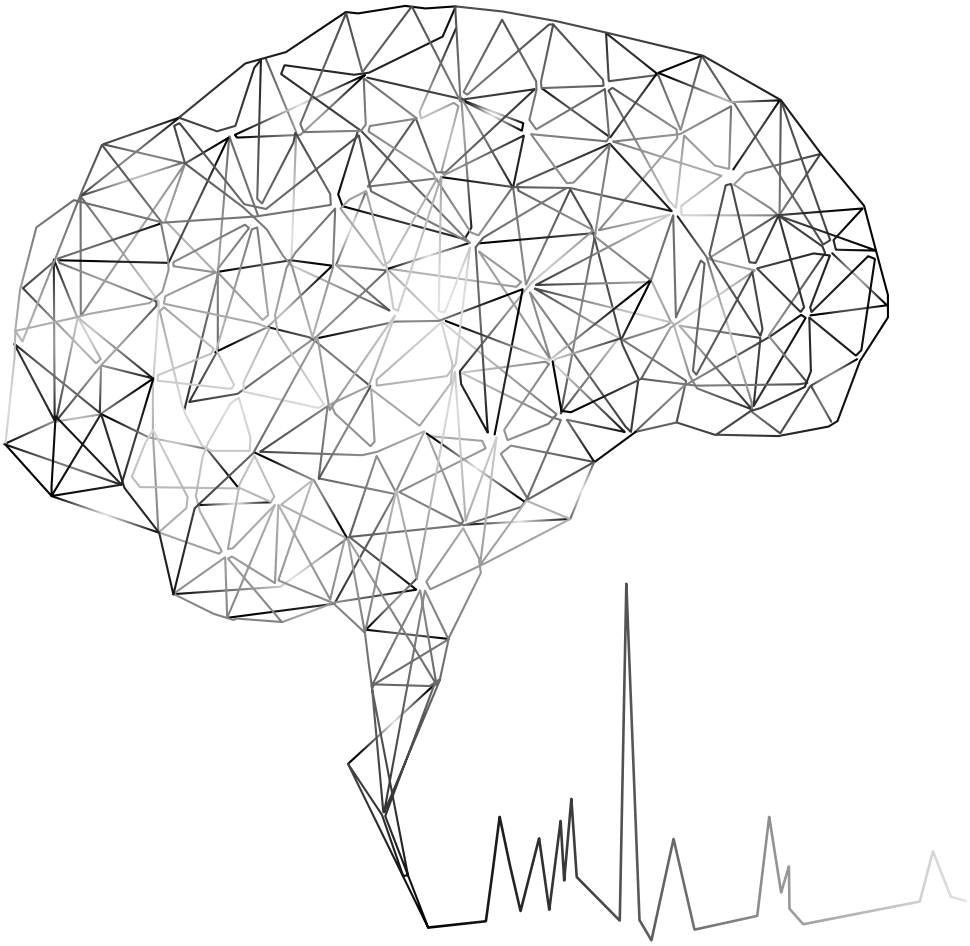
Radboud Alzheimer Center series | Donders Graduate School for Cognitive Neuroscience



NEDERLANDSE SAMENVATTING

Inleiding

De ziekte van Alzheimer is de meest voorkomende oorzaak van dementie bij ouderen. Wereldwijd zijn er meer dan 20 miljoen mensen met deze vorm van dementie. Bij de ziekte van Alzheimer krijgen mensen problemen met hun geheugen, met het plannen van activiteiten, met hun taalgebruik en met het vinden van de weg in hun omgeving. Ondanks de enorme inzet van onderzoekers is genezing van de ziekte van Alzheimer onmogelijk. Er wordt daarom veel onderzoek gedaan naar leefstijlfactoren, omdat het kansen biedt voor het voorkomen van deze ziekte. Voeding is bijvoorbeeld een leefstijlfactor waarvan we weten dat het de kans op het krijgen van de ziekte van Alzheimer, of dementie in het algemeen, kan vergroten of verkleinen. Er is veel interesse in de vraag of en hoe voedingsstoffen het beloop of ontstaan van de ziekte van Alzheimer kunnen beïnvloeden. Met name de verbindingen tussen hersencellen (synapsen) zijn daarbij interessant: hoe goed die verbindingen functioneren is namelijk mogelijk te beïnvloeden met voedingsstoffen. Dit komt doordat de bouwstenen van de verbindingen continu aangemaakt en afgebroken worden en er dus zo continu een behoefte aan bouwstenen bestaat. Het belangrijkste component van de bouwstenen zijn de fosfolipiden en de continue opbouw en afbraak daarvan noemen we fosfolipidenmetabolisme. Wij krijgen fosfolipiden binnen door het eten van bijvoorbeeld vette vis.

Of we nu voeding eten of medicijnen innemen: om de cognitieve symptomen van de ziekte van Alzheimer te kunnen voorkomen, verminderen of omkeren, moet een interventie zijn weg vinden naar de hersenen via de (bloed)circulatie. Alleen dan kan het klinisch beloop veranderd worden. In dit proefschrift heb ik onderzocht wat het effect van verschillende voedingsstoffen (nutriënten) op de hersenen van mensen met de ziekte van Alzheimer is en of de voedingsstoffen in de circulatie terecht komen. Hierbij heb ik me met name gericht op nutriënten die betrokken zijn bij het fosfolipidenmetabolisme. De meest gebruikte techniek in dit proefschrift is magnetische resonantie spectroscopie (MRS), een techniek waarmee we metabolieten in de hersenen kunnen onderzoeken op een niet-invasieve manier, vergelijkbaar met MRI. Zo kunnen we o.a. de metabolieten onderzoeken die een rol spelen bij het fosfolipidenmetabolisme. Daarnaast krijgen we informatie over het energiemetabolisme. Hieronder volgt een samenvatting van de bevindingen.

Het effect van voedingsstoffen op de (bloed)circulatie en de hersenen.

In hoofdstukken 2 en 4 onderzochten we het effect van een interventie die meerdere nutriënten bevat (een multi-nutriënte interventie), bij mensen met de ziekte van Alzheimer. Deze multi-nutriënte combinatie bevat voedingstoffen die een rol spelen bij de aanmaak en afbraak van fosfolipiden. In hoofdstuk 2 analyseerden we de gegevens van meer dan 900 mensen met de ziekte van Alzheimer die eerder hadden meegedaan aan drie grote studies naar het effect van deze interventie op o.a. het geheugen. Deze patiënten gebruikten 24 tot 48 weken lang elke

dag de multi-nutriënte combinatie of een controle product. In het bloed van de patiënten zagen we dat de niveaus van de nutriënten die in het geteste product zaten (vitamines, mineralen en vetzuren) na 6 weken, 12 weken en 24 weken gestegen waren. Als het product nog langer gebruikt werd, dan bleven de niveaus stabiel. In een deel van de patiënten hebben we ook gekeken of er een effect was op ontstekingswaarden in het bloed of tekenen van oxidatieve stress, maar dit was niet het geval in deze studie.

Nadat we in hoofdstuk 2 hadden laten zien dat de voedingsstoffen uit het product een effect hadden op de bloedwaarden, gingen we in hoofdstuk 4 een stap verder. We onderzochten of er ook een effect was op de hersenen bij mensen met de ziekte van Alzheimer. In deze studie hebben we patiënten (in het beginstadium van de ziekte) gevraagd om 4 weken lang elke dag de multi-nutriënte combinatie of een controleproduct te gebruiken. Vooraf en na deze periode hebben we met MRS het fosfolipidenmetabolisme en de neurale integriteit onderzocht. We zagen dat de verhouding tussen de bouwstenen van fosfolipiden en de afbraakproducten van fosfolipiden verhoogd was in de hersenen na 4 weken. Daarmee hebben we laten zien dat we in korte tijd het fosfolipidenmetabolisme (een essentieel metabool proces) in de hersenen kunnen beïnvloeden met een voedingsinterventie. We konden echter niet aantonen dat ook de neurale integriteit veranderd was in deze korte tijd.

Fosfolipiden- en energiemetabolisme in de ziekte van Alzheimer

Omdat er onvoldoende informatie was over het fosfolipidenmetabolisme bij mensen met de ziekte van Alzheimer, onderzochten we dit in hoofdstuk 3 met behulp van de laatste ontwikkelingen op het gebied van (fosfor) MRS. Tegelijkertijd gaven deze metingen ook informatie over het energiemetabolisme van de hersenen. Studies van het brein van mensen met de ziekte van Alzheimer die waren overleden hadden veranderingen in fosfolipiden laten zien. Wij zagen echter geen verschillen in het fosfolipidenmetabolisme tussen patiënten in het beginstadium van de ziekte van Alzheimer en een groep van even oude gezonde deelnemers. We zagen wel veranderingen in het energiemetabolisme. De interessantste bevinding was dat het belangrijke energiemolecuul fosfocreatine verhoogd was. Die verhoging zagen we in die delen van het brein die al vroeg zijn aangedaan in de ziekte van Alzheimer, maar niet in een ander deel van het brein dat pas later betrokken raakt bij de ziekte.

Voedingssupplementen als aanvulling op medicatie.

In hoofdstuk 5 bekeken we in een uitgebreid literatuuronderzoek of voedingssupplementen iets toevoegen aan de traditionele behandeling met medicatie (choline-esterase remmers zijn de meest gebruikte medicijnen bij de ziekte van Alzheimer). Hierbij keken we dit keer naar effecten op cognitief functioneren (o.a. geheugen) en dagelijks functioneren (o.a. zelfstandig kunnen wassen, aankleden, eten). We zagen dat de toevoeging van vitamine E-supplementen aan choline-esterase remmers van alle onderzochte supplementen als enige de achteruitgang in dagelijks functioneren kon vertragen. De cognitieve achteruitgang werd echter niet beïnvloed

door de toevoeging van vitamine E of enig ander voedingssupplement. Hoewel we weten dat voedingssupplementen het risico op de ziekte van Alzheimer kunnen verkleinen en effect hebben op het lichaam en de hersenen, laat deze studie zien dat het erg lastig blijkt om in patiënten met de ziekte van Alzheimer te zorgen voor een cognitieve of functionele verbetering.

Conclusies

Met de studies in dit proefschrift onderzocht ik de effecten van nutriënten in de ziekte van Alzheimer, met een focus op die nutriënten die betrokken zijn bij het fosfolipidenmetabolisme. We toonden aan dat zowel nutriëntenniveaus in de (bloed)circulatie als essentiële metabole processen in het brein beïnvloed kunnen worden door multi-nutriënte supplementatie, die bouwstenen voor de aanmaak van fosfolipiden bevat. Uiteindelijk zou dit cognitieve achteruitgang kunnen verminderen door de ondersteuning van het functioneren van synapsen. Echter, uit een literatuuroverzicht bleek dat er op dit moment geen bewijs is dat de toevoeging van voedingssupplementen aan standaard Alzheimer-medicatie de cognitieve achteruitgang van patiënten vertraagt. Het huidige onderzoek heeft ook geleid tot de ontdekking van afwijkingen in het energiemetabolisme in de ziekte van Alzheimer, waarbij er sprake is van een specifiek anatomisch patroon. Met toekomstige technologische vooruitgangen in (fosfor) MR, zou fosfocreatine kunnen uitgroeien tot een nieuwe MR-biomarker voor de ziekte van Alzheimer.

LIST OF ABBREVIATIONS

^{1}H	proton
^{31}P	phosphorus
A-A	active-active
ACC	anterior cingulate cortex
AD	Alzheimer's disease
ADAS-cog	Alzheimer's Disease Assessment Scale, cognitive subdomain
ADAS-noncog	Alzheimer's Disease Assessment Scale, non-cognitive subdomain
ADCS-ADL	Alzheimer's Diseae Cooperative Study Activities of Daily Living
ADP	adenosine diphosphate
AE	adverse event
ANCOVA	analysis of covariance
ApoE	apolipoprotein E
ATP	adenosine triphosphate
BADL	Basic Activities of Daily Living
BMI	body mass index
C-A	control-active
CAS	Caregiver Activity Survey
CASI	Cognitive Abilities Screening Instrument
CDR	Clinical Dementia Rating scale
CGIC	Clinical Global Impression of Change
ChE-I	cholinesterase inhibitor
CK	creatine kinase
Cr	creatine
CRP	C-reactive protein
CSF	cerebrospinal fluid
DHA	docosahexaenoic acid
DPA	docosapentaenoic acid
DSST	Digit Symbol Substitution Test
EPA	eicosapentaenoic acid
FC	Fortasyn Connect
GM	grey matter
GPCh	glycerophosphocholine
GPEth	glycerophosphoethanolamine
Hcy	homocysteine
HL	left hippocampus
HPLC	high-performance liquid chromatography
HR	right hippocampus
IADL	Instrumental Activities of Daily Living

IL	interleukin
ITT	intention to treat
LS	least square
MCI	mild cognitive impairment
MDA	malondialdehyde
mI	*myo*-inositol
MMSE	Mini Mental State Examination
MRS	magnetic resonance spectroscopy
MRSI	magnetic resonance spectroscopic imaging
n-3 LC-PUFA	n-3 long-chain polyunsaturated fatty acids
NAA	*N*-acetyl aspartate
NAD(H)	nicotinamide adenine dinucleotide
ND	not done
NOSGER	Nurses Observation Scale for Geriatric patients
NS	non-significant
OLE	open-label extension
OXPHOS	oxidative phosphorylation
PC	phosphatidylcholine
PCh	phosphocholine
PCr	phosphocreatine
PDE	phosphodiesters
PE	phosphatidylethanolamine
PET	positron emission tomography
PEth	phosphoethanolamine
Pi	inorganic phosphate
PME	phosphomonoesters
RCT	randomized controlled trial
RDA	recommended dietary allowance
ROI	region of interest
RSC	retrosplenial cortex
SB	Social Behavior
SD	standard deviation
SEM	standard error of the mean
tCho	total choline
tCr	total creatine
TE	tocopherol equivalents
TMT	Trail Making Test
UK	unknow
UMP	uridine monophosphate
WM	white matter

LIST OF PUBLICATIONS

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ABOUT THE AUTHOR

Anne Rijpma was born in Hengelo, the Netherlands, on October 28, 1985. In 2005 she started her bachelor study Psychology at the Radboud University in Nijmegen, with a specialization in Neuropsychology and Rehabilitation Psychology. Having caught the research bug during her student assistant's job at the Max Planck Institute for Psycholinguistics, she successfully applied for the research Master's program Cognitive Neuroscience at the Donders Institute for Brain, Cognition and Behaviour in Nijmegen. She received her Master's degree (cum laude) in 2011 and started her PhD project at the department of Geriatric Medicine, Radboud university medical center, in 2012. Under supervision of professor Olde Rikkert (Geriatric Medicine) and professor Heerschap (Biomedical MR) she performed the research described in this thesis. She currently works on MRI in Alzheimer's disease in the research group of dr. Claassen at the department of Geriatric Medicine.

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