

# Treatment of Alzheimer's Disease with a Cholinesterase Inhibitor Combined with Antioxidants

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## Key Words

Alzheimer's disease · d-ROMs test · Glutathione · Homocysteine · Sickle erythrocytes

## Abstract

A formula (formula F) was prepared to counteract oxidative stress (OS) in the brain. The formula contained the most common antioxidants and was intended to: (a) protect proteins, lipids, DNA and proteoglycans from oxidation (carnosine, coenzyme Q<sub>10</sub>, vitamin E, vitamin C, β-carotene, selenium, L-cysteine and ginkgo biloba); (b) reduce homocysteine (HCy) blood levels (vitamins B<sub>6</sub>, B<sub>9</sub> and B<sub>12</sub>), and (c) sustain the pentose phosphate cycle in circulating cells (vitamins B<sub>1</sub>, B<sub>2</sub> and B<sub>3</sub>). Formula F contained low doses of each antioxidant component and was administered in a two-phase ampoule. A cohort of 52 patients (21 males and 31 females) affected with moderate probable AD (according to NINCDS-ARDA and NINCS-AIREN criteria) already being treated with donepezil (5 mg/day for at least two months) was randomly divided into two groups, and followed for 6 months. A double-blind design was used in which 26 cases were treated once a day with formula F plus donepezil, and the other 26 with placebo plus donepezil. The level of OS was measured on the basis of a d-ROMs test (which measures plasma hydroperoxides), plasma HCy and glutathione, and percentage

of sickle erythrocytes. The two patient groups were comparable for all variables (age, sex, concomitant diseases, ApoE ε4, MMSE II score, OS, antioxidant reserve and sickle erythrocytes). Forty-eight subjects completed the trial. Significant decreases in OS and HCy were only observed when there was an increase in glutathione (in erythrocytes) and a decrease in sickle erythrocytes in patients treated with formula F. The MMSE II score remained almost the same in the group treated with donepezil and placebo, whereas some significant improvements were found in the group treated with donepezil plus formula F. Copyright © 2010 S. Karger AG, Basel

## Introduction

The brain has certain characteristics that make it very sensitive to oxidative stress (OS). Although it accounts for 2% of body weight, the brain consumes 20% of the total O<sub>2</sub> inhaled. It also has the highest concentration of docosahexaenoic acid, an unsaturated fatty acid that is extremely sensitive to oxidation. The iron concentration in some areas of the brain, such as the red nucleus and basal ganglia, lays the ground for substantial oxidation. This is also supported by the reactive oxygen species (ROS) generated by dopamine auto-oxidation.

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Another peculiarity of the brain is its lack of a lymphatic system, which means that tissue drainage has to take place through the blood vessels because CSF circulation is too low (25 ml/h) to support this task.

All these characteristics make the brain very sensitive to OS, which may be one of the causes of many degenerative diseases [1–3], especially Alzheimer's disease (AD). It is not enough, however, to administer one or two antioxidants at high doses to improve the antioxidant network in the brain. It is necessary to consider many aspects of oxidation. At least six elements take part in the OS process in AD: vessels/blood brain barrier (BBB), mitochondria, homocysteine (HCy), amyloid, erythrocytes and proteoglycans (PGs).

The brain vessels do not support blood flow to tissue efficiently in AD. The brain's attempt to compensate with vasodilatation is clear from the increase in nitric oxide production. This can be seen from the dramatic increase in dimethylargininase, which is one of the key enzymes in nitric oxide production [4–7]. However, the concomitant presence of superoxide ( $O_2^{\cdot-}$ ), owing to local inflammatory processes, generates peroxynitrate ( $ONOO^-$ ) and hydroxyl radicals ( $OH^{\cdot}$ ), which may cause vasoconstriction [8, 9] when produced in large quantities. The brain vasculature (about 600 km of vessels) ends up at the BBB, which consists of many components (endothelial cells, pericytes, neuron feet, astrocytes, etc.) connected in a highly coordinated structure. It is so coordinated that it has to be considered a true neurovascular unit [10, 11]. Because of its nature, lesion of one component directly affects all the others. Consequently, the BBB is one of the first structures damaged in AD.

The second structures on the list are mitochondria. BBB endothelial cells are characterized by tight junctions that require large quantities of energy to keep the barrier effective. Consequently many mitochondria are found in the cells to provide the necessary ATP. Another source of this high energy production is the presence of ROS, which can only be sufficiently quenched by a very efficient antioxidant system [12]. There are at least seven different systems that produce ROS in mitochondria, and any alterations to the respiratory chain cytochromes end up in two parallel phenomena: a decrease in ATP and an increase in ROS production [13–15]. ATP may be imported from the cytoplasm to compensate for the shortage in energy production. If this is not possible, the cell undergoes apoptosis.

At least three mitochondrial systems relating to energy production have been shown to be deficient in AD: pyruvate dehydrogenase,  $\alpha$ -ketoglutarate dehydroge-

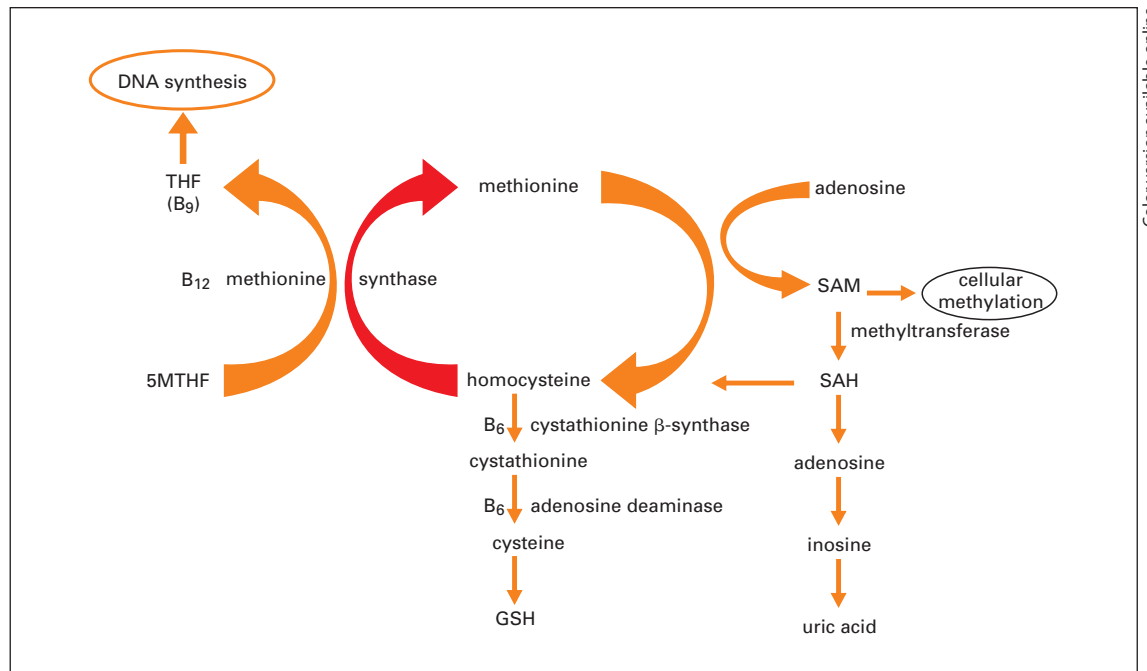
nase, and cytochrome oxidase [16, 17]. Mitochondrial abnormalities related to OS have been described in neurons, lymphocytes [18], endothelial cells [11] and fibroblasts [19] of subjects affected with AD. This condition may be one of the main causes of the loss in efficiency of endothelial cell function and consequently of BBB activity [20, 21].

The third element is HCy. An increase in HCy plasma levels has been correlated with alterations in the EEG of patients with AD [22] and is a known cause of endothelial damage [23, 24]. HCy is produced during metabolism in strict connection with methionine, cellular methylation, DNA and glutathione (GSH) synthesis. A few details of the HCy metabolic pathway are needed to point out the key steps in reducing its plasma levels (fig. 1).

HCy turnover is very complex and related to many different activities. Supplementation with vitamins B<sub>6</sub>, B<sub>9</sub> and B<sub>12</sub> or even B<sub>6</sub> alone has been found to reduce HCy levels [25–27]. Some clinical trials have shown that cardiovascular mortality is not affected by the use of the vitamin B group despite the decrease in HCy levels [28, 29]. However, the doses administered to patients were extremely high: 40 mg of vitamin B<sub>6</sub> and 0.4 mg of vitamin B<sub>12</sub>, i.e. 20 and 200 times the RDA, respectively. These amounts may cause damaging effects in the long-term.

The fourth important factor is amyloid formation from amyloid precursor protein [30, 31]. Peptides A $\beta$  1–40/42 have both antioxidant and pro-oxidant activity, but they are definitely pro-oxidant [32, 33] and, when produced in large amounts, cause vasoconstriction in cerebral vessels [34]. The pro-oxidant activity damages proteins by forming advanced glycated end products and protein cross-linking. Carnosine may be used to protect against this specific brain oxidation [35, 36]. The first aggregate forms of A $\beta$  peptides are soluble dimers and trimers [37, 38] which are neurotoxic. The oligomers and fibrillar forms, on the other hand, seem to lack both oxidant activity and neurotoxicity. As a consequence, brain cell proteins have to be protected from the damage caused mainly by the initial aggregation of A $\beta$  peptides (2–4 peptides).

This is the task of erythrocytes, which are the fifth important element in brain protection. The main activity of erythrocytes is hemoglobin O<sub>2</sub> transport. This task is done very efficiently since each erythrocyte contains about 300 million hemoglobin molecules. All the hemoglobin is protected from oxidation basically by the NAD(P)H obtained through the pentose phosphate cycle.



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**Fig. 1.** Metabolic pathway of HCy. HCy is methylated by 5-methyltetrahydrofolate (5MTHF) in the presence of vitamin B<sub>12</sub> to form tetrahydrofolate (THF), the active form of vitamin B<sub>9</sub>. If there is a shortage of 5MTHF, DNA/RNA synthesis is impaired. Methionine is needed to produce S-adenosyl methionine (SAM), which is the main cellular methyl donor. HCy is also produced by S-adenosyl HCy (SAH). The cysteine which is derived from HCy in the presence of vitamin B<sub>6</sub> is needed to form GSH. Adapted from Pogribna et al. [78].

In this cycle (fig. 2), glucose is activated to glucose 6 phosphate (G6P) by ATP and becomes ribulose 5 phosphate (R5P), with the presence of vitamin B<sub>1</sub> as a cofactor. This allows the production of four ATP molecules.

GSH is produced from NAD(P)H and is used to protect the hemoglobin from oxidation and to conjugate toxic products, which are exported from erythrocytes by active pumps [39, 40].

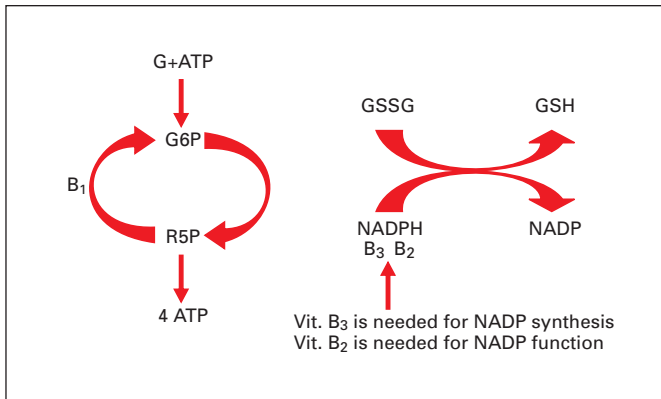
Vitamins B<sub>3</sub> and B<sub>2</sub> are needed as cofactors to produce NADP and activate this process. Since large quantities of GSH are needed when erythrocytes undergo OS, they can produce tripeptides from single amino acids (L-cysteine, glycine and glutamine) and also need selenium (Se) to produce the 'anti-stress' enzyme glutathione peroxidase (GPx 1). GPx 1 contains a particular GSH formed with Se-L-cysteine instead of L-cysteine as a prosthetic part.

Erythrocytes bind amyloid dimers or trimers [41, 42] that generate pore-like structures on the erythrocyte membrane and can alter the cell membrane and volume. They exhibit ionophore-like properties [43] and undergo OS, resulting in an increase in erythrocyte size and the

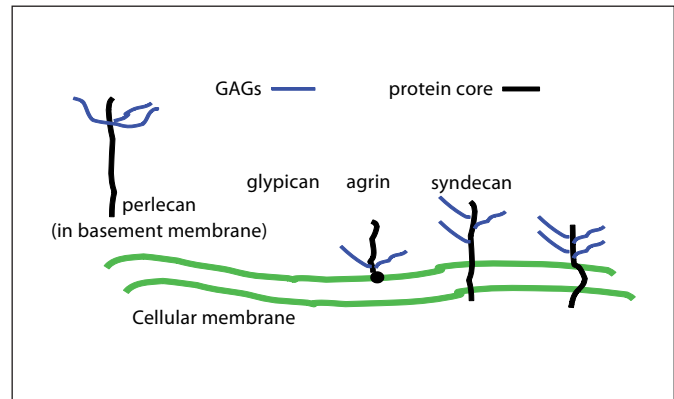
assumption of an elongated shape (sickle erythrocytes). In these OS conditions, erythrocytes may deposit their amyloid burden on endothelial cells [44]. In patients suffering from AD, sickle erythrocytes increase in number until reaching around 20%, whereas in comparable control cases it is far less than 10% [41].

The sixth important element in the development of AD is proteoglycans (PGs). PGs are found both on cells and in the extracellular matrix. They consist of a few glycosaminoglycans (GAGs) linked to a core protein, and between 100,000 and 1 million of them are found on every cell. The core proteins differ according to the type of PG, but they are usually large proteins (>500 kDa). However, both core proteins and GAGs may have highly variable dimensions.

GAGs are usually highly sulphated and consist of heparan sulphate, dermatan sulphate, chondroitin sulphate or keratan sulphate. All these molecules consist of chains of disaccharides, either glucose or galactose derivatives [45–47], and the chains are made up of polymers of several saccharides (usually >50). PGs are chemically well defined and have different names, such as perlecan, glyp-



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**Fig. 2.** Pentose phosphate cycle and vitamins B<sub>1</sub>, B<sub>2</sub> and B<sub>3</sub>. Part of the R5P is also used for trioses, which generate four molecules of ATP, and part returns to G6P. During the passage from G6P to R5P, a reducing unit (NADPH) is generated, which donates hydrogen in order to transform oxidized glutathione (GSSG) into GSH.

**Fig. 3.** PGs are a sort of signal indicating the receptor. The GAG is specific for the ligand that interacts with the receptor. The GAGs found on PGs are negatively charged owing to the presence of many sulphate groups on the saccharides, and may drive different ligands by charge repulsion or charge attraction. Fibroblast growth factors for instance only bind to their receptor after dimerization triggered by the heparan sulphate of the PG.

ican, syndecan, agrin, etc. They differ from the core protein and according to the type and number of GAGs. The location of PGs is also characteristic; perlecan is only found in the basement membrane of the BBB and glomeruli, whereas agrin and syndecan are very often found in BBB endothelial cells. Figure 3 shows some PGs and their respective locations on the cellular membrane.

Oxidized PGs [48, 49] have to be internalized for substitution. Owing to the high negative charge of their GAGs, they can attract all compounds with positive charges, such as A $\beta$  peptides. This charge binding can trigger amyloid formation inside endothelial cells (vascular amyloid) or within neurons and astrocytes (cerebral amyloid).

The formation of neurofibrillary tangles (NFTs) is also related to the PGs internalized for substitution, as shown in figures 4 and 5. The internal aggregation of hyperphosphorylated tau protein with PG interferes with microtubule repair (fig. 4). This aggregation is self-maintaining up to the formation of a NFT (fig. 5) and any attempt to repair microtubules ends up with a sort of 'abortion'.

Basically, the process starts with the aggregation of hyperphosphorylated tau protein triggered by the internalized PGs that have to be substituted for repair.

In conclusion, damaged PGs are involved both in the formation of amyloid and NFTs. These are the most important markers of neuronal damage in Alzheimer's disease, seen in the form of neuritic and/or senile plaques.

### Brain Antioxidant: Formula F

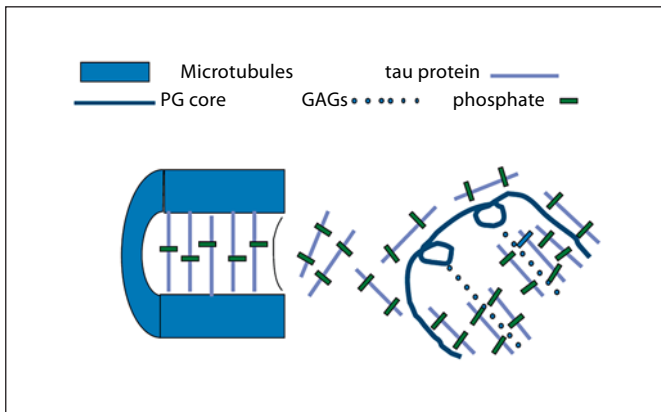
Considering all aspects of OS in the brain (BBB, mitochondria, HCy, amyloid, erythrocytes and PGs), a specific antioxidant formula (table 1) was created to improve the antioxidant network in subjects suffering from AD.

The components were combined according to the following concepts: carnosine to protect proteins from amyloid oxidation, which produces advanced glycated end products and cross-linking; vitamins B<sub>1</sub>, B<sub>2</sub> and B<sub>3</sub> to support the pentose phosphate cycle in erythrocytes and brain cells; vitamins B<sub>6</sub>, B<sub>9</sub>, B<sub>12</sub> to reduce HCy plasma levels; Se and L-cysteine to increase GSH production [50–52]; coenzyme Q<sub>10</sub> to improve mitochondrial function [53]; vitamin E,  $\beta$ -carotene and vitamin C to protect cell membranes from oxidation; ginkgo biloba to support the vasodilatation of micro- and macrovessels and for its antioxidant activity [54–56].

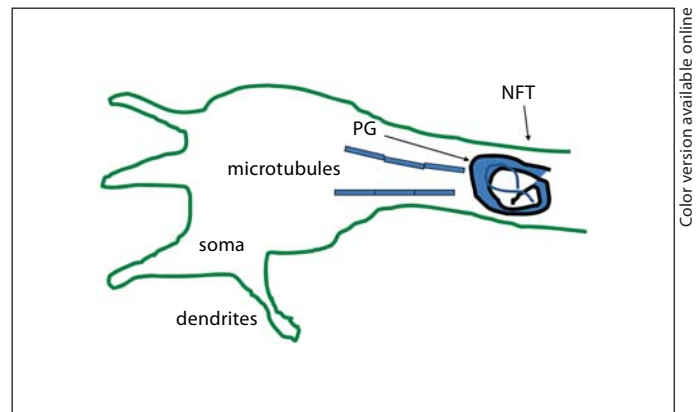
We tested formula F in patients in a controlled, clinical study. The aim of the double-blind clinical trial was to treat patients suffering from mild AD in combination with donepezil for a period of 6 months, and to evaluate changes in plasma OS.

### Materials and Methods

A cohort of 52 patients suffering from probable AD was randomly divided into two groups and followed for a period of 6



**Fig. 4.** Components of an NFT: hyperphosphorylated tau protein, PGs and amyloid. Tau protein becomes hyperphosphorylated to repair microtubules and its aggregation is triggered by PGs, which also impair microtubule repair.



**Fig. 5.** Formation of NFTs in neurons. Microtubules transport substances from the soma of a neuron to its periphery (dendrites or synapses) and vice versa, and the initial NFT formation does not allow them to be repaired.

**Table 1.** Formula to protect brain from aging: formula F (AR<sub>D</sub> Cogiton<sup>®</sup>)

Antioxidant	Dose	RDA
Carnosine	100 mg	–
Thiamine (B <sub>1</sub> )	1.4 mg	100
Riboflavin (B <sub>2</sub> )	1.6 mg	100
Nicotinamide (B <sub>3</sub> )	18 mg	100
Pyridoxine (B <sub>6</sub> )	2 mg	100
Folic acid (B <sub>9</sub> )	200 µg	100
Cyanocobalamin (B <sub>12</sub> )	1 µg	100
Vitamin C	30 mg	50
Vitamin E	20 mg	200
Coenzyme Q <sub>10</sub>	10 mg	–
β-Carotene	800 RE	100
Selenium	27.5 µg	25
L-cysteine	10 mg	–
Ginkgo biloba	25 mg	–

months according to a double-blind design. A group of 26 subjects was treated with formula F ampoules, and a similar group of 26 cases with placebo ampoules. Treating AD patients with placebo only (without any specific AD treatment) was not allowed by the ethics committee. Consequently, the experiment was performed on patients already being treated with donepezil. All the subjects were referred to a UVA centre (Unit for Alzheimer' Disease Evaluation and Assistance) and the protocol was approved by the center's ethics committee.

#### Admission Criteria

Cases were diagnosed according to NINCDS-ADRDA [57] and NINDS-AIREN [58]. Patients with an MMSE II score >21

with diagnosis of probable AD were admitted, provided they had been treated with donepezil at 5 mg/day for at least 2 months and had been receiving stable treatment (for at least 3 months) for any other concomitant chronic diseases. The type of ApoE ε alleles was not considered as an inclusion/exclusion criterion but was simply measured as a routine variable.

#### Exclusion Criteria

Patients not assisted by a caregiver, with malignancies or receiving chemotherapy were not admitted. Patients with an MMSE II score <21, alcohol abuse or severe depression were excluded. Patients being treated with donepezil for less than 2 months or with dosages of donepezil of >5 mg/day were also excluded.

A patient register was kept in order to record the number of subjects who referred to the centre during the enrolment period and the reason for their exclusion. Enrolment was completed in less than 2 weeks. One hundred and twenty patients were considered and 52 cases complied with the admission criteria.

Most of the exclusions were related to the severity of the disease (MMSE II score <21), the short period of donepezil treatment (<2 months) or because patients were undergoing treatment with drugs other than donepezil. It was decided to exclude subjects being treated with drugs other than donepezil because their interactions with the antioxidants may be different and this might lead to misinterpretation of results.

#### Treatments

All patients were undergoing treatment with 5 mg of donepezil. One group was treated with antioxidant formula F at a dose of one ampoule/day in the morning immediately before breakfast, and the other group was treated with placebo (excipients plus 500 mg of fructose and flavoring) at one ampoule/day. The placebo ampoule was identical to the formula F ampoule.

All treatments were administered for a period of 6 months. A 3-month supply of either formula F or placebo was given to each patient (9 boxes of 10 ampoules each). Compliance with the antioxidant treatment was measured by counting the remaining am-

**Table 2.** Characteristics of patients treated with formula F and placebo

Variables	Formula F	Placebo	p <sup>1</sup>
Sex			NS
Males	9	10	
Females	14	15	
Age	75 ± 4.2	74 ± 4.9	NS
Hypertension	6	5	NS
NIDD	6	5	NS
MI	5	6	NS
Hyperlipidemia	6	6	NS
ApoE ε4 <sup>2</sup>	4	5	NS

NIDD = Non-insulin-dependent diabetes; MI = previous myocardial infarction (at least 3 years before). Some patients were also affected with other chronic diseases (urological/gynecological), but their frequencies were too low to be evaluated statistically.

<sup>1</sup>  $\chi^2$  test with Yates correction or t test for independent data; NS = p > 0.05.

<sup>2</sup> At least one ε4 allele.

poules returned by the subject during the checks. Compliance was also checked for donepezil on the basis of a tablet count (four boxes of 28 tablets were given to each patient). No other treatments were checked for compliance.

After the first 3-month period, patients were asked to refer to the centre again for an intermediate check and treatment supply.

#### *Principal and Secondary Parameters*

The main parameter considered was oxidative status measured using the d-ROMs test [59, 60] on plasma. HCy, plasma GSH levels, and the percentage of sickle erythrocytes were considered as secondary parameters. The overall clinical condition measured with MMSE II score was also considered as a secondary parameter.

#### *Laboratory Assessment and Rating Scales*

At each check (baseline, 3 months and 6 months), blood samples were collected in heparinized and nonheparinized tubes in the morning (between 8 and 10 a.m.) after overnight fasting. Three different aliquots of 5 ml each (two for plasma and one for serum) were taken from the brachial vein. They were immediately centrifuged and kept at -80°C until the measurements were taken. One further aliquot of 2 ml was also taken (without heparin) for sickle erythrocyte analysis at baseline and after 6 months only.

The d-ROMs test, erythrocyte GSH [61] and plasma HCy [62] were measured as described elsewhere. The d-ROMs test was performed at each check, whereas all the other tests were done at baseline and after 6 months only.

To measure the percentage of sickle erythrocytes, an erythrocyte pellet was isolated by centrifugation (1,000 g for 15 min at 4°C) immediately after the collection of a 2-ml sample in 2 ml citrate-phosphate-dextrose solution with adenine. The top of the

tube was discarded and the process was repeated three times using CDPA for resuspension. Immediately after preparation, the percentage of sickle erythrocytes was calculated manually (on 400 erythrocytes) using Nomarski optics [41].

An MMSE II was performed at baseline and after 3 and 6 months, no more than 1 day before or after blood sampling. A change of at least 1 point in the MMSE II score compared with the baseline value was considered clinically relevant both in terms of improvement ( $\geq +1$ ) and worsening ( $\leq -1$ ).

Sleeping was also measured using a three-point scale (no change, better or worse).

#### *Sample Size and Statistical Analysis*

The sample size was based on the expected decrease in plasma hydroperoxides measured using the d-ROMs test, which is a test for measuring OS. Preliminary experiments on patients suffering from AD in non-blind conditions (2 months of treatment) gave some indication of the expected d-ROMs test values. An average decrease of at least 70–80 ± 10 (SD) CARR.U. was expected in the treated group (corresponding to a decrease of about 20% compared with the baseline value), whereas the placebo group was expected to show no more than a 20 ± 10 CARR.U. decrease (corresponding to a decrease of about 5% of the baseline value).

For a value of  $\alpha = 0.05$  and  $1-\beta = 0.9$ , groups of 20 subjects are sufficient to obtain a power of at least >0.9. Assuming a maximum dropout of 30%, a total number of 26 patients were added to each group. Average values ± SD were calculated for all variables. The t tests for independent and interdependent data were used in order to measure the differences between baseline values and final values and between treatments, respectively.

Correlation coefficients were calculated between all the variables, and the exact  $\chi^2$  (Fisher) was used to measure the improvement/worsening in MMSE II score.

## **Results**

Two subjects in the group treated with formula F underwent major orthopedic surgery a few weeks after starting the trial and were excluded from the evaluation.

One patient treated with formula F and one with placebo did not come to the first check (after 3 months) and were excluded from the analysis. Consequently, 48 of the initial 52 cases concluded the trial: 23 cases with formula F and 25 with placebo.

The general characteristics of the patients are shown in table 2.

The antioxidant treatment was very well tolerated, and no relevant side effects that could be interpreted as due to the antioxidant treatment were reported by any group member. Compliance was excellent since the remaining dose count was in line with expected values. None of the concomitant chronic diseases (hypertension, diabetes type II, hyperlipidemia, etc.) was affected by the treatments.

The main results are shown in tables 3 and 4. The baseline data and data after 6 months of treatment are shown for the variables assessed in the laboratory (table 3).

A significant reduction in OS was only found in subjects treated with the antioxidant. As regards the OS values recorded after 3 months of treatment, only the d-ROMs test data were available for all patients. In a few cases, other variables were also available (data not shown).

The measurements relating to the MMSE II score recorded at baseline, 3 and 6 months are shown in table 4.

The average improvement in MMSE II score was not very substantial in either group. However, considering each case singularly, an improvement in MMSE II score ( $\geq +1$ ) was found in 12 patients in the formula F group and in only 4 cases treated with placebo. This difference is statistically significant according to the Fisher test ( $p < 0.05$ ).

A fall ( $\leq -1$ ) in MMSE II score was also observed in one case treated with the antioxidant and 2 cases treated with placebo. These numbers are too small to draw conclusions.

The correlation coefficients between the differences in the values recorded at baseline and after 6 months were calculated for the group treated with antioxidant supplementation (table 5). The same calculation was not possible for the placebo group because there were not enough cases of improvement for this type of analysis.

Significant correlation was found between the MMSE II differential (6 months – baseline) and the decrease in sickle erythrocytes. The increase in GSH was also strongly correlated with the improvement in MMSE II score. The decrease in d-ROMs test values was correlated with both the decrease in sickle erythrocytes and increase in GSH. The latter two variables also showed significant internal correlation ( $p < 0.01$ ).

All the variables were tested for correlation and against each other at baseline, using the combined data from both groups. None of the variables were significantly correlated.

## Discussion

Since all the patients were being treated with donepezil, the results obtained from this experiment have to be considered as the combined effect of a cholinesterase inhibitor with antioxidant supplementation.

One initial limitation of this trial stems from the type of patients included, since the diagnosis of AD was concomitant with other chronic diseases which may all also

**Table 3.** Laboratory assessment of patients suffering from AD

Variables	Period	Formula F	Placebo
d-ROMs test, CARR.U.	baseline	380 ± 44.6	365 ± 37.8
	6 months	295 ± 26.3 <sup>1,2</sup>	356 ± 40.2
HCy, µmol/l	baseline	27 ± 5.4	29 ± 5.9
	6 months	20 ± 2.9 <sup>1,2</sup>	27 ± 2.3
GSH, µmol/ml	baseline	2.6 ± 0.72	2.9 ± 0.84
	6 months	3.2 ± 0.82 <sup>1</sup>	3.0 ± 0.79
Sickle erythrocytes, % <sup>3</sup>	baseline	18 ± 4.4	20 ± 4.2
	6 months	12 ± 3.8 <sup>1,2</sup>	17 ± 3.7 <sup>1</sup>

Values shown are mean ± SD.

<sup>1</sup> t test  $p < 0.05$  for dependent data (before vs. 6 months).

<sup>2</sup> t test  $p < 0.05$  for independent data (formula F vs. placebo at 6 months).

<sup>3</sup> Measurement in the group treated with formula F was only performed in 20 subjects.

**Table 4.** MMSE II score in the two patient groups (mean ± SD)

Treatment	Period	MMSE II	Cases improved/worsened
Donepezil + formula F	baseline	23.2 ± 1.14	
	3 months	24.0 ± 1.57	4/0
	6 months	24.3 ± 1.43	11*/1
Donepezil + placebo	baseline	23.9 ± 1.04	
	3 months	23.6 ± 1.11	0
	6 months	24.2 ± 1.28	4/2

\*  $p < 0.05$ , exact  $\chi^2$  test (Fisher), formula F vs. placebo.

**Table 5.** Correlation coefficients for all variables in the group treated with antioxidant formula

Variables	MMSE II	d-ROMs test	HCy	Sickle erythrocytes	GSH
MMSE II	1				
d-ROMs test	0.388	1			
HCy	0.242	-0.194	1		
Sickle erythrocytes <sup>1</sup>	0.816**	0.489*	0.179	1	
GSH	0.881**	0.499*	0.307	0.621**	1

\*  $p < 0.05$ ; \*\*  $p < 0.01$ .

<sup>1</sup> Measurement was done in only 20 cases.

cause OS. The improvement shown by the antioxidants administered in formula F may not be specifically related to AD, but to the other diseases as well. Furthermore, the sample size was sufficient for the d-ROMs test (the principal variable), but was not big enough to detect any significant change in the MMSE II score with sufficient power. In this case, the number of patients needed would be much greater. Sickle erythrocytes were studied in relation to amyloid only [41, 42], but theoretically their increase could be detected in many other diseases. Therefore, to draw correct clinical conclusions, more studies will have to be conducted in this area.

Despite all these limitations, formula F treatment was shown to substantially reduce OS and to improve the clinical results obtained with donepezil. Consequently, all the differences in the results of the two groups may be attributed to the antioxidant treatment.

The data showing a decrease in the number of sickle erythrocytes are particularly interesting. These data show that oxygen transport to the brain is greater owing to the presence of more deformable erythrocytes, thus with positive consequences on microcirculation. This effect could be very positive in the case of high levels of amyloid in the vasculature, as usually occurs even before AD symptoms become evident.

The antioxidant treatment was found to improve the antioxidant network substantially as shown by the increase in plasma GSH. This is mainly a measure of the antioxidant capability of circulating cells and especially erythrocytes, which make up the majority. This effect acts in parallel with the decrease in sickle erythrocytes.

These two tests were found to be highly correlated with the improvement in MMSE II score. Plasma GSH is merely a biochemical measurement, whereas the number of sickle erythrocytes is more related to function. The correlation between these two parameters indicates that the increase in circulating antioxidant may improve erythrocyte membrane function. Consequently, the whole brain may benefit from higher O<sub>2</sub> availability and this may have a favorable impact on cognitive function.

HCy was not found to be related to clinical outcome measured in terms of MMSE II score. This lack of correlation between HCy and clinical improvement was also found by other authors for cardiovascular diseases [28, 29]. One explanation might be that HCy is only one of many epiphenomena of a more complex equilibrium with other inflammatory markers [63], and that its decrease is due to a more general reduction in OS and not specifi-

cally to microvessel endothelial cell stress. This may explain why some authors found that its reduction was concomitant with a decrease in the risk of stroke [64], and that short-term treatment is effective on carotid intima-media thickness, whereas long-term treatment is not [65]. However, regardless of its origin, HCy can be a cytotoxic oxidant [66] and its decrease helps quench OS. All known HCy metabolic pathways were considered in the current formulation, especially its transformation into GSH through efficient NAD(P)H turnover. This was done by administering a more complete vitamin B group and not just vitamins B<sub>6</sub>, B<sub>9</sub>, and B<sub>12</sub> alone.

This is the first time that a combination of antioxidants at doses within the RDA has been found to be active in decreasing OS in patients with AD. Excessive doses of vitamin E and vitamin C are usually administered [67, 68], which may behave as pro-oxidants [69]. Supplementation with vitamin B<sub>6</sub> and vitamin B<sub>12</sub> at high doses [70] with or without vitamin B<sub>9</sub> [71] was also found to be ineffective in the treatment of cognitive decline. Combinations of different types of antioxidant at lower doses (within the RDA) should be tested with the aim of improving the whole antioxidant network.

The variability in the intake of antioxidants with diet makes comparing clinical data derived from different countries very complex. Patients should use antioxidants according to their needs as determined by measuring OS. The amount of oxidized vitamin E, for instance, was recently found to be directly related to the severity of cognitive impairment [72].

Despite the need for further larger studies, the idea of simultaneously tackling brain oxidation in many different compartments seems to be promising.

Donepezil has been found to be active in the treatment of cognitive impairment [73, 74], but association with vitamin E at high doses has not been seen to improve its activity [75]. In contrast, the larger spectrum of antioxidants at doses close to the RDA used in this trial was found to be helpful. Treatment with these types and doses of antioxidants could be even more useful in preventing/delaying the onset of the disease in those patients who have risk factors without any overt AD symptoms.

Age is the most important risk factor. The risk of AD doubles for every 5 years of age increase, and millions of people in every developed country are at risk of this disease, which is becoming a real economic problem for all societies. There is no alternative to aging. Aging instead can be faced with some preventive measures. In other words, when a subject is 60 years old and has some risk



factors, he should be carefully monitored for the additional risk of OS and particular importance should be placed on its measurement.

Further studies with a larger population and involving other types of AD therapy are needed to clarify the

role of antioxidants in the treatment of AD. Furthermore, more specific tests for brain oxidation [76, 77] should become available for routine studies in the field to understand the real clinical value of reducing brain oxidation.

## References

- Ding Q, Dimayuga E, Keller JN: Oxidative damage, protein synthesis, and protein degradation in Alzheimer's disease. *Curr Alzheimer Res* 2007;4:73–79.
- Dröge W, Schipper MH: Oxidative stress and aberrant signaling in aging and cognitive decline. *Aging Cell* 2007;6:361–370.
- Christen Y: Oxidative stress and Alzheimer disease. *Am J Clin Nutr* 2000;71:621S–629S.
- Smith MA, Vasak K, Knipp M, et al: Dimethylargininase, a nitric oxide regulatory protein, in Alzheimer's disease. *Free Radic Biol Med* 1998;25:898–902.
- Luth HJ, Munch G, Gartner U, et al: Expression of endothelial and inducible NOS-isoforms is increased in Alzheimer's disease, in APP23 transgenic mice and after experimental brain lesions in rats: evidence for an induction by amyloid pathology. *Brain Res* 2001;913:57–67.
- Heneka MT, Wiesinger H, Dumitrescu-Ozimek L, et al: Neuronal and glial coexpression of arginosuccinate synthetase and inducible nitric oxide synthase in Alzheimer disease. *J Neuropathol Exp Neurol* 2001;60:906–916.
- Lee SC, Zhao ML, Hirano A, Dickson DW: Inducible nitric oxide synthase immunoreactivity in the Alzheimer disease hippocampus: association with Hirano bodies, neurofibrillary tangles, and senile plaques. *J Neuropathol Exp Neurol* 1999;58:1163–1169.
- Soneja A, Drews M, Malinski T: Role of nitric oxide, nitroxidative and oxidative stress in wound healing. *Pharmac Report* 2005;57:108–119.
- Malinski T: Nitric oxide and nitroxidative stress in Alzheimer's disease. *J Alzheimers Dis* 2007;11:207–218.
- Hawkins BT, Davis T: The blood-brain barrier/neurovascular unit in health and disease. *Pharmacol Rev* 2005;57:173–185.
- Stewart PA, Hayakawa K, Akers MA, Vinters HV: A morphometric study of the blood-brain barrier in Alzheimer's disease. *Lab Invest* 1992;67:734–742.
- Andreyev AY, Kushnareva YE, Starkov AA: Mitochondrial metabolism of reactive species. *Biochemistry (Moscow)* 2005;70:200–214.
- Wang J, Xiong S, Xie C, et al: Increased oxidative damage in nuclear and mitochondrial DNA in Alzheimer's disease. *J Neurochem* 2005;93:933–962.
- Lin MT, Beal MF: Mitochondrial dysfunction and oxidative stress in neurodegenerative diseases. *Nature* 2006;433:787–795.
- Sas K, Robotka H, Toldi J, Vécsei L: Mitochondria, metabolic disturbances, oxidative stress and kynurenine system, with focus on neurodegenerative disorders. *J Neurol Sci* 2007;257:221–239.
- Marlatt M, Lee H, Perry G, et al: Sources and mechanism of cytoplasmic oxidative damage in Alzheimer's disease. *Acta Neurobiol Exp* 2004;64:81–87.
- Hirai K, Aliev G, Nunomura A, et al: Mitochondrial abnormalities in Alzheimers disease. *J Neurosci* 2001;21:3017–3023.
- Mecocci P, Polidori MC, Cherubini A, et al: Lymphocyte oxidative DNA damage and plasma antioxidants in Alzheimer disease. *Arch Neurol* 2002;59:794–798.
- Blass JP, Gibson GE: The role of oxidative abnormalities in the pathophysiology of Alzheimer's disease. *Rev Neurol (Paris)* 1991;147:513–525.
- Zhu X, Smith MA, Honda K, et al: Vascular oxidative stress in Alzheimers disease. *J Neurol Sci* 2007;257:240–246.
- Hachinski V, Munoz DG: Cerebrovascular pathology in Alzheimer's disease: cause, effect or epiphenomenon? *Ann NY Acad Sci* 1997;826:1–6.
- Babiloni C, Bosco P, Ghidoni R, et al: Homocysteine and electroencephalographic rhythms in Alzheimer disease: a multicentric study. *Neuroscience* 2007;143:942–954.
- Seshadri S, Beiser A, Selhub J, et al: Plasma Hcy as risk factor for dementia and Alzheimer's disease. *NEJM* 2002;346:476–483.
- Ravaglia G, Forti P, Maioli F, et al: Hcy and folate as risk factors for dementia and Alzheimer disease. *Am J Clin Nutr* 2005;82:636–643.
- Bleie Ø, Refsum H, Ueland PM, et al: Changes in basal and postmethionine load concentration of total Hcy and cystationine after B vitamin intervention. *Am J Clin Nutr* 2004;80:641–648.
- Lee BJ, Huang MC, Chung LJ, et al: Folic acid and vitamin B<sub>12</sub> are more effective than vitamin B<sub>6</sub> in lowering fasting plasma homocysteine concentration in patients with coronary artery disease. *Eur J Clin Nutr* 2004;58:481–487.
- NcKinley MC, McNulty H, McPartlin J, et al: Low-dose vitamin B<sub>6</sub> effectively lowers fasting Hcy in healthy elderly person who are folate and riboflavin replete. *Am J Clin Nutr* 2001;73:759–764.
- Bonaa KH, Njølstad NJ, Ueland PM, et al: Homocysteine lowering and cardiovascular events after acute myocardial infarction. *NEJM* 2006;354:1578–1588.
- Ebbing M, Bleie Ø, Ueland PM, et al: Mortality cardiovascular events in patients treated with Hcy-lowering B vitamin after coronary angiography: a randomized clinical trial. *JAMA* 2008;300:795–804.
- Selkoe DJ: Normal and abnormal biology of the  $\beta$ -amyloid precursor protein. *Annu Rev Neurosci* 1994;17:489–517.
- Näslund J, Haroutonian V, Mohs R, et al: Correlation between elevated levels of amyloid  $\beta$ -peptide in the brain and cognitive decline. *JAMA* 2000;283:1571–1577.
- Butterfield DA: Amyloid beta-peptide (1–42)-induced oxidative stress and neurotoxicity: implication for neurodegeneration in Alzheimer's disease brain. A review. *Free Radic Res* 2002;36:1307–1313.
- Kontush A: Amyloid-beta: an antioxidant that becomes a pro-oxidant and critically contributes to Alzheimer's disease. *Free Radic Biol Med* 2001;31:1120–1131.
- Suo Z, Su G, Kundtz A, et al: A beta vasoactivity in vivo. *Ann NY Acad Sci* 2000;903:156–163.
- Guiotto A, Calderan A, Ruzza P, Borin G: Carnosine and carnosine-related antioxidants: a review. *Curr Med Chem* 2005;12:2293–2315.
- Hipkiss AR: Could carnosine or related structures suppress Alzheimer's disease? *J Alzheimers Dis* 2007;11:229–240.
- Cleary JP, Walsh DM, Hofmeister JJ, et al: Natural oligomers of the amyloid-beta protein specifically disrupt cognitive function. *Nat Neurosci* 2005;8:79–84.
- Walsh DM, Selkoe DJ: Oligomers on the brain: the emerging role of soluble protein aggregates in neurodegeneration. *Protein Pept Lett* 2004;11:213–228.

- 39 Alhamdani MSS: Impairment of glutathione biosynthetic pathway in uremia and dialysis. *Nephrol Dial Transplant* 2005;20:124–128.
- 40 Reid M, Badaloo A, Forrester T, Jahoor F: In vivo rates of erythrocytes glutathione synthesis in adults with sickle cell disease. *Am J Physiol Endocrinol Metab* 2006;291:E73–E79.
- 41 Mohanty JG, Eckley DM, Williamson JD, et al: Do red blood cell- $\beta$ -amyloid interaction alter oxygen delivery in Alzheimer's disease? *Adv Exp Med Biol* 2008;614:29–35.
- 42 Singer SJ, Dewji NN: Evidence that Perutz's double- $\beta$ -stranded subunit structure for  $\beta$ -amyloid also applies to their channel-forming structures in membranes. *PNAS* 2006;103:1546–1550.
- 43 Engström I, Ronquist G, Petterson L, Waldenström A: Alzheimer amyloid beta-peptides exhibit ionophore-like properties in human erythrocytes. *Eur J Clin Invest* 1995;25:471–476.
- 44 Ravi LB, Mohanty JG, Chrest FJ, et al: Influence of beta-amyloid fibrils on the interactions between red blood cells and endothelial cells. *Neurol Res* 2004;26:579–585.
- 45 Cornelli U: Glycosaminoglycans as potential future treatment of Alzheimer's disease; in Richter RW, Richter BZ (eds): *Alzheimer's Disease. A Physician Guide to Practical Management*. Totowa, Humana Press, 2004.
- 46 Van Vactor D, Wall DP, Johnson KG: Heparan sulphate proteoglycans and the emergence of neuronal connectivity. *Curr Opin Neurobiol* 2006;16:50–51.
- 47 Galtrey CM, Fawcett JW: The role of chondroitin sulphate proteoglycans in regeneration and plasticity in the central nervous system. *Brain Res Rev* 2007;54:1–18.
- 48 Metcalfe DD, Thompson HL, Klebanoff SJ, Henderson WR: Oxidative degradation of rat mast-cell proteoglycan. *Biochem J* 1990;272:51–57.
- 49 Roberts CR, Roughley PJ, Mort JS: Degradation of human proteoglycan aggregates induced by hydrogen peroxide. *Biochem J* 1989;259:805–811.
- 50 Wessjohann LA, Schneider A, Abbas M, Brandt W: Selenium in chemistry and biochemistry in comparison to sulfur. *Biol Chem* 2007;388:997–1006.
- 51 Wu G, Fang YZ, Yang S, et al: Glutathione metabolism and its implications for health. *J Nutr* 2004;134:489–492.
- 52 Franco R, Schoneveld OJ, Pappa A, Panayiotidis MI: The central role of glutathione in the pathophysiology of human diseases. *Arch Physiol Biochem* 2007;113:234–258.
- 53 Littarru GP, Tiano L: Bioenergetic and antioxidant properties of coenzyme Q10: recent developments. *Mol Biotechnol* 2007;37:31–37.
- 54 Koltermann A, Harkorn A, Koch E, et al: Ginkgo biloba extract Egb 761 increases endothelial nitric oxide production in vitro and vivo. *Cell Mol Life Sci* 2007;64:1715–1722.
- 55 Napryeyenko O, Borzenko I, GINDEM-NP Study Group: Ginkgo biloba special extract in dementia with neuropsychiatric features. A randomized placebo-controlled double-blind clinical trial. *Arzneimittelforschung* 2007;57:4–11.
- 56 Mazza M, Capuano A, Bria P, Mazza S: Ginkgo biloba and donepezil: a comparison in the treatment of Alzheimer's dementia in a randomized placebo-controlled double-blind study. *Eur J Neurol* 2006;13:981–985.
- 57 McKhann G, Drachman D, Folstein M, et al: Clinical diagnosis of Alzheimer's disease: report of the NINCDS-ADRDA Work Group under auspices of Department of Health and Human Services Task Force on Alzheimer's Disease. *Neurology* 1984;34:939–944.
- 58 Román GC, Tatemichi TK, Erkinjuntti T, et al: Vascular dementia: diagnostic criteria for research studies: report of the NINDS-AIREN International Workshop. *Neurology* 1993;43:250–260.
- 59 Cesarone MR, Belcaro G, Carratelli M, et al: A simple test to monitor oxidative stress. *Int Angiol* 1999;18:127–130.
- 60 Cornelli U, Terranova R, Luca S, et al: Bio-availability and antioxidant activity of some food supplements in men and women using the D-Roms test as a marker of oxidative stress. *J Nutr* 2001;131:3208–3211.
- 61 Reid M, Badaloo A, Forrester T, Jahoor F: In vivo rates of erythrocyte glutathione synthesis in adults with sickle cell disease. *Am J Physiol Endocrinol Metab* 2006;291:E73–E79.
- 62 Bleie Ø, Refsum H, Ueland PM, et al: Changes in basal and postmethionine load concentration of total homocysteine and cystathionine after B vitamin intervention. *Am J Clin Nutr* 2004;80:641–648.
- 63 McDermott MM, Liu K, Ferrucci L, et al: Circulating markers and functional impairment in peripheral arterial disease. *J Am Geriatr Soc* 2008;56:1504–1510.
- 64 Wang X, Qin X, Demitras H, et al: Efficacy of folic acid supplementation in stroke prevention: a meta analysis. *Lancet* 2007;369:1876–1882.
- 65 Potter K, Hankey GJ, Green DJ, et al: The effect of long term homocysteine-lowering on carotid intima-media thickness and flow-mediated vasodilation in stroke patients: a randomized controlled trial and meta-analysis. *BMC Cardiovasc Disord* 2008;8:24.
- 66 Stanger O, Herrmann W, Pietrzik K, et al: DACH-LIGA Homocystein (German, Austrian and Swiss Homocysteine Society): consensus paper on the rational clinical use of homocysteine, folic acid and B-vitamins in cardiovascular and thrombotic disease: guidelines and recommendation. *Clin Chem Lab Med* 2003;41:1392–1403.
- 67 Yaffe K, Clemons TE, McBee WL, et al: Impact of antioxidants, zinc, and copper, on cognition in the elderly: a randomized clinical trial. *Neurology* 2004;63:1705–1707.
- 68 Gray SL, Anderson ML, Crane PK, et al: Antioxidant vitamin supplement use and risk of dementia or Alzheimer's disease in older adults. *J Am Geriatr Soc* 2008;56:291–295.
- 69 Miller ER 3rd, Pastor-Barriuso R, Dalal D, et al: Meta-analysis: high dosages vitamin E supplementation may increase all-cause mortality. *Ann Intern Med* 2005;142:37–46.
- 70 Aisen PS, Schneider LS, Sano M, et al: High-dose B vitamin supplementation and cognitive decline in Alzheimer disease: a randomized controlled trial. *JAMA* 2008;300:1774–1783.
- 71 Malouf M, Grimley EJ, Areosa SA: Folic acid with or without vitamin B<sub>12</sub> for cognition and dementia. *Cochrane Database Syst Rev* 2003;CD004514.
- 72 Ravaglia G, et al: Plasma tocopherol and risk of cognitive impairment in an elderly Italian cohort. *Am J Clin Nutr* 2008;87:1306–1313.
- 73 Ringman JM: Donepezil improves cognitive function and reduces decline in activities of daily living in people with severe Alzheimer's disease. *Evid Based Ment Health* 2006;9:104–117.
- 74 Setzer B: Donepezil an update. *Expert Opin Pharmacother* 2007;8:1011–1023.
- 75 Petersen RC, Thomas RG, Grundman M, et al: Vitamin E and donepezil for the treatment of mild cognitive impairment. *NEJM* 2005;352:2379–2388.
- 76 Montine TJ, Montine KF, McMahan W, et al: F<sub>2</sub>-isoprostanes in Alzheimer and other neurodegenerative diseases. *Antiox Redox Signal* 2005;7:269–275.
- 77 Montine TJ, Quinn J, Kaie J, Morrow JD: F(2)-isoprostanes as biomarkers of late-onset Alzheimer's disease. *J Mol Neurosci* 2007;33:114–119.
- 78 Pogribna M, Melnik S, Pogribni I, Chango P, et al: Hcy metabolism with Down syndrome: in vitro stimulation. *Am J Hum Genet* 2001;69:88–95.