ALZHEIMER'S DISEASE AND RELATED DISORDERS - ORIGINAL ARTICLE

Intake of copper has no effect on cognition in patients with mild Alzheimer's disease: a pilot phase 2 clinical trial

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Abstract Disturbed copper (Cu) homeostasis may be associated with the pathological processes in Alzheimer's disease (AD). In the present report, we evaluated the efficacy of oral Cu supplementation in the treatment of AD in a prospective, randomized, double-blind, placebo-controlled phase 2 clinical trial in patients with mild AD for 12 months. Sixty-eight subjects were randomized. The treatment was well-tolerated. There were however no significant differences in primary outcome measures (Alzheimer's Disease Assessment Scale, Cognitive subscale, Mini Mental Status Examination) between the verum [Cu-(II)-orotate-dihydrate; 8 mg Cu daily] and the placebo group. Despite a number of findings supporting the hypothesis of environmental Cu modulating AD, our results demonstrate that oral Cu intake has neither a detrimental nor a promoting effect on the progression of AD.

Keywords Cu · Alzheimer · Clinical trial · ADAS-cog · MMSE

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Abbreviations

AAS	Atomic absorption spectroscopy
$A\beta$	Amyloid beta peptide
AD	Alzheimer's disease
ADAS-cog	Alzheimer's disease assessment scale, cognitive subscale
ADRDA	Alzheimer's disease and related disorders association
APP	Amyloid precursor protein
CDT	Clock drawing test
Cu	Copper
CuBD	Copper binding domain
MMSE	Mini mental status examination
NINCDS	National Institute of Neurological and Communicative Disorders and Stroke
SOD-1	Superoxide dismutase-1
Zn	Zinc

Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disease characterized by extracellular deposition of

Aβ peptides in senile plaques and intracellular accumulation of hyperphosphorylated τ protein in neuronal cells as neurofibrillary tangles.

Potentially toxic Aβ peptides are generated from the copper-binding amyloid precursor protein (APP) by two independent proteolytic events (Bayer et al. 2001; Glenner and Wong 1984; Hesse et al. 1994; Kang et al. 1987). APP is actively involved in balancing Cu concentrations in cells. In APP-knock-out mice, Cu levels were found increased in cerebral cortex and liver (White et al. 1999), whereas overexpression of APP was reported to result in significantly reduced Cu levels in brain tissue of different APP transgenic mouse strains (Bayer et al. 2003; Phinney et al. 2003) and in mice overexpressing the C-terminal fragment of APP (and enhanced Aβ secretion) (Maynard et al. 2002).

The N-terminal Cu binding domain (CuBD-I) of APP shows structural homology to the Cu binding domain of Cu chaperons (Barnham et al. 2003) binding Cu with nanomolar affinity (Hesse et al. 1994). A secondary CuBD-II appears in Aβ after its release from APP (Atwood et al. 2000), and Cu application was reported to increase Aβ aggregation in vitro [reviewed in (Bush 2003)]. APP reduces Cu (II) to Cu (I), leading to oxidative modification of APP (Multhaup et al. 1996), which is facilitated through the protein surface localization of the binding site thus resembling so-called cytoplasmic Cu chaperones (Barnham et al. 2003).

In cell culture systems, Cu supplementation was found to stimulate the non-amyloidogenic APP pathway thereby suppressing the formation of β amyloid (Borchardt et al. 1999). More recently, APP was shown in yeast cells to have a Cu efflux activity thereby explaining why APP overexpressing mice have a reduced Cu level in their brains (Bayer et al. 2003; Phinney et al. 2003; Treiber et al. 2004).

In the brain, APP transgenic mice have not only lower Cu levels but they also exhibit a reduced Cu, Zn superoxide dismutase-1 (SOD-1) activity compared to wild-type mice. Dietary Cu supplementation in a transgenic mouse model for AD increased bioavailable brain Cu levels, restored SOD-1 activity, prevented premature death and decreased Aβ levels (Bayer et al. 2003). In Wilson's disease, a mutation of copper ATPase 7B leads to Cu accumulation in the liver and a threefold to fourfold higher Cu level in the brain. After crossbreeding of APP transgenic mice with so-called toxic milk mice having a defect in the copper ATPase 7B it was observed that APP-related lethality could be rescued. In addition, Aβ levels were significantly reduced due to the genetically upregulated Cu level (Phinney et al. 2003).

Earlier studies in animals have reported that elevated Cu is a risk factor for developing the AD related pathology.

Cherny et al. (2001) showed that clioquinol, a copper and zinc chelating agent, can remove β amyloid plaque pathology. However, it was unclear how this effect worked, since the authors reported an increase of soluble Cu and Zn levels in the brain of treated mice. This apparently contradictory finding could be explained by the finding that clioquinol mediates Cu uptake by transporting Cu across cell membranes counteracting Cu efflux activities of APP (Treiber et al. 2004).

Normally, Cu contained in the food is taken up in the stomach and then absorbed in the small intestine. About 30–50% of the Cu is absorbed. Cu is distributed from the liver throughout the body and transported in the bloodstream bound to ceruloplasmin. The liver is the most important organ for Cu distribution and storage. Cu is excreted via the biliary system. Usually, 2 mg of Cu per day are taken with food. Ingestion of as much as 10 mg of Cu per day is considered to be safe. The clinical reference value for physiological Cu plasma concentrations is 65–165 µg/dl [reviewed in (Kessler et al. 2005)].

Since increased Cu levels reduced Aβ production in APP transgenic mouse models, we speculated that Cu intake stabilizes cognitive decline in AD patients. Thus, we treated AD patients with supplemental Cu and monitored the progression of the disease by recording the cognitive performance for 12 months using neuropsychological tests.

Material and methods

Study population

Criteria for participation in the study included written informed consent as well as caregiver consent, a diagnosis of probable AD by means of NINCDS-ADRDA criteria (McKhann et al. 1984), a Mini Mental Status Examination score <25, and receipt of a 5–10 mg dose of donepezil for at least 2 months. All patients continued to receive donepezil during the study. To be enrolled in the study patients had to be 50–80 years of age and not able to bear children. Patients with severe and unstable somatic diseases and patients with present or known history of alcohol, drug or medication abuse were excluded. Patients taking drugs for coexistent diseases were included except those taking psychotropic drugs, "nootropics" or health food supplements. The trial has been approved by the appropriate ethics committee and has therefore been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki. All persons gave their informed consent prior to their inclusion in the study.

Study design

The study had a monocenter, prospective, double-blind, placebo-controlled, parallel-group randomized design. Sixty-eight patients were recruited. Patients were randomly allocated either to placebo or to verum (Cu orotate). The oral dosage of Cu-(II)-orotate-dihydrate was 51.62 mg (corresponding to 8 mg Cu) once daily. To maintain blinding, capsules containing Cu orotate and placebo had identical shape and color. The duration of the study was 12 months. The study was registered at www.clinicaltrials.gov with the identification number NCT00608946.

Study procedures

Screening procedures consisted of a medical history, physical examination and psychometric tests. Blood was collected to determine the level of Cu and zinc by using atomic absorption spectroscopy (AAS). Furthermore, blood count included liver enzymes and anti-mitochondrial antibodies (for exclusion of cholestatic liver diseases). Atomic absorption spectroscopy: Blood samples were collected into metal-free tubes that contained lithium heparin as an anticoagulant. Concentrations of Cu were measured in lithium-heparin plasma samples, utilizing flame AAS (Perkin Elmer, AAnalyst 800). Samples were diluted with deionized water and the analysis was performed against standards prepared in glycerol to approximate the viscosity characteristics of the diluted samples. Standard atomic absorption conditions were utilized for Cu (air acetylene, wave length 324.8 nm).

Outcome measures

The primary efficacy variables were the changes from the baseline score on the cognitive subscale of the Alzheimer's Disease Assessment Scale (ADAS-cog) and on the MMSE. Efficacy evaluations were performed at baseline and at month 3, 6, 9 and 12.

Therapeutic drug monitoring

Plasma Cu levels were measured at every visit (at baseline and at month 3, 6, 9 and 12).

Safety measures

Standard adverse event reporting was conducted. Before baseline, the Cu content in drinking water at the patients' home was analyzed under standard conditions; concentration of Cu in drinking water must not exceed 2 mg/l. For safety reasons, blood analyses (including especially plasma Cu levels and liver enzymes) were performed at week 1, 2, 3 and 4 after baseline and at month 3, 6, 9 and 12.

Data analysis

Evidence of efficacy was indicated by a significant difference in change from baseline between treatment arms. For statistical analyses SPSS 14 was used. All tests were two-tailed. Demographic variables, data on the course of the disease and clinical data at baseline of the patients that completed the examinations were compared between the verum and the placebo group with one-way analysis of variance (ANOVA). Chi-square test on independence was used to analyze, if the distribution of sex was different between the two groups. Dependent variables were the ADAS-cog total score and the MMSE score at baseline (t_0) and at four following measurements with intervals of 3 months each (t_3, t_6, t_9, t_{12}). Furthermore, plasma Cu levels at the beginning (t_0) and at the end (t_{12}) of the study were compared. Independent between-subject factor was treatment group (placebo, verum), within-subject factor was time of measurement. For ADAS-cog, MMSE and plasma copper the differences between the values at the end (t_{12}) and at the beginning of the study (t_0) were computed as measures for the changes over time. Pearson's product moment correlations between these differences and intervening variables age, disease duration, age at onset of the disease and duration of education were calculated, intending to analyze, if these variables show a significant influence on the changes of the dependent variables. ANOVA with between-subject factors treatment group and gender was use to analyze, if the changes were gender-related. For the dependent variables Kolmogorov–Smirnov tests were used to test, if there were significant deviations from the normality assumption. The results of these preliminary statistics regulated the main analysis. The basic hypotheses were that the dependent variables ADAS-cog and MMSE change continuously over time and that these changes are different between the placebo and the verum group. The third hypothesis was that plasma copper level increases over time in the verum, but not in the placebo group. To analyze these hypotheses, the general linear model procedure was used to perform multivariate analyses of variance (MANOVA) with repeated measures design. Within-subject factor was time of measurement between-subject factor was treatment group. Since there was no significant influence of the intervening variables sex, age, disease duration, age at onset of the disease and duration of education on the differences of the dependent variables between the values at the end and at the beginning of the study, no additional covariates or factors were entered to the model in the main analysis. In the repeated measures analyses MANOVA performed an orthonormal transformation of the dependent variables. The Mauchly test of sphericity was used to test the assumption that the covariance matrix of the transformed orthonormal variables was
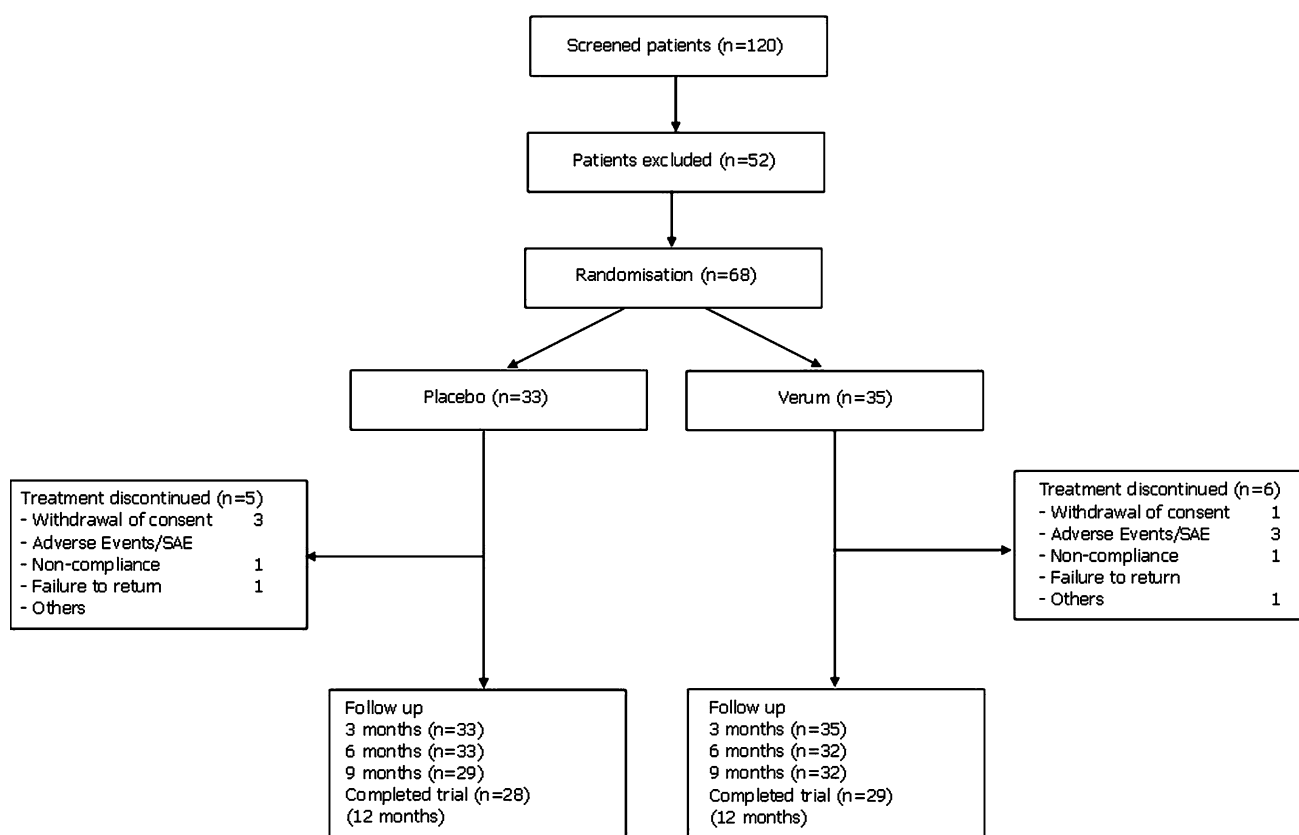
Fig. 1 Outline of flowchart of subjects studied. *SAE* serious adverse event

constant on the diagonal and zero off the diagonal. This was the case, if the values of the dependent variables show the same error and interaction effects at each measure time. If the sphericity assumption was violated, the Greenhouse–Geisser epsilon correction was applied.

Results

Subject recruitment and demographics

The randomization of patients and their progress through the study is summarized in Fig. 1. A total of 120 patients were screened; 52 of these patients were excluded due to exclusion criteria. Altogether 68 subjects were randomized, with 33 patients receiving placebo and 35 patients receiving Cu. There were no significant mean differences for age, disease duration, age at onset of the disease, education and clock drawing test at baseline between the verum and the placebo patients. The sex distribution was not significantly different between the two treatment groups (for details see Table 1). For the total verum and placebo group there were no significant correlations between the intervening variables age, disease duration, age at onset of the disease, duration of education and the changes over time for

ADAS cog ($t_{12} - t_0$), MMSE ($t_{12} - t_0$) and plasma copper ($t_{12} - t_0$). From ANOVA with factors group and gender there were no significant gender effects on the changes of the dependent variables over time. Therefore, no intervening variables had to be entered to the model in the main analysis. For all dependent variables, Kolmogorov–Smirnov tests did show no significant deviations from normality assumption.

Proof of concept

For the ADAS-cog score, an increase of the scores over time was observed in the placebo group (t_{12} vs. t_0: +15.5%) and in the verum group (t_{12} vs. t_0: +8.8%). From MANOVA with the repeated measures design there were significant effects of within-subject factor time ($df = 3.3$, 177.1; $F = 3.1$; $P = 0.023$) and of between-subject factor treatment group ($df = 1$, 53; $F = 4.7$; $P = 0.034$) pointing out generally lower values in the verum group. However, there was no significant time × treatment group interaction ($df = 3.3$, 177.1; $F = 0.4$; $P = 0.78$) (Fig. 2a).

The MMSE score, a less sensitive measure of cognitive impairment, showed a similar pattern. There was a decrease over time in both treatment groups (t_{12} vs. t_0: placebo: −9.5%, verum: −10.5%). The MANOVA

Table 1 Demographic and clinical data at baseline of the patients that completed the study

Sex (no. of males; no. of females)	Placebo ($n = 28$) m: 10; f: 18 Mean $\pm$ SD	Verum ($n = 29$) m: 15; f: 14 Mean $\pm$ SD	df 1 df	χ^2 1.48 F	P 0.23 P
Age (years)	69.4 $\pm$ 8.1	69.6 $\pm$ 6.6	1, 55	0.01	0.94
Disease duration (months)	25.2 $\pm$ 16.2	34.0 $\pm$ 31.2	1, 54	1.76	0.19
Age at onset (years)	67.4 $\pm$ 7.9	66.6 $\pm$ 6.1	1, 54	0.17	0.68
Education (years)	10.6 $\pm$ 1.9	11.6 $\pm$ 2.9	1, 54	2.28	0.14
CDT (scores)	2.8 $\pm$ 1.3	2.8 $\pm$ 1.2	1, 55	0.01	0.93

n Number of cases, SD standard deviation, $no.$ number, F F statistics, χ^2 χ^2 statistics, P error probability for falsely rejecting the null hypothesis, that there are no mean differences between the treatment groups

repeated measures analysis resulted in significant effects of within-subject factor time ($df = 3.2$, 173.8; $F = 9.5$; $P < 0.0005$). Effects of between-subject factor treatment group were not significant ($df = 1$, 54; $F = 1.6$; $P = 0.21$), and there was no significant interaction between time and treatment group ($df = 3.2$, 173.8; $F = 0.2$; $P = 0.88$) (Fig. 2b).

Altogether, cognitive abilities worsened progressively in AD patients receiving Cu as well as in those patients taking placebo. Differences between the two groups in the mean change from baseline scores were not statistically significant at any visit.

Plasma levels of copper

In both groups, mean absolute levels of Cu at baseline were within the age-related normative range. From MANOVA repeated measures analysis for plasma copper there were no significant mean differences for within-subject factor time ($df = 1$, 50; $F = 1.7$; $P = 0.20$) and no significant effects for between-factor treatment group ($df = 1$, 50; $F = 1.1$; $P = 0.29$), while the time $\times$ treatment group interaction was significant ($df = 1$, 50; $F = 5.2$; $P = 0.027$), as mean plasma Cu was decreased over time in the placebo group (t_{12} vs. t_0: t_{12} mean $\pm$ SD $= 98.5 \pm 17.7$ µg/dl and t_0 mean $\pm$ SD $= 109 \pm 23.4$ µg/dl; -7.5%), but stabilized in the verum group (t_{12} vs. t_0: t_{12} mean $\pm$ SD $= 100.8 \pm 21.6$ µg/dl and t_0 mean $\pm$ SD $= 100.7 \pm 15.3$ µg/dl; $+2.3\%$). No treatment effect was however observed on Zn and ceruloplasmin levels (not shown).

Safety results and analysis

Safety analysis was conducted on all data irrespective of the stage reached in the trial. In the placebo group, 28 patients (84.5%) continued taking part until the end of the study, in the verum group 29 patients (82.9%) completed treatment. From the Chi-square test on independence, the drop-out rate was not statistically different between the two groups ($\chi^2 = 0.05$, $P = 0.82$). Six patients discontinued the

treatment in the Cu group: one withdrew consent, three due to adverse events, one due to non-compliance and one patient for other reasons. The mentioned drop-outs related to serious adverse events were hospitalizations due to intracranial hemorrhage (1 patient), diagnosis of non-Hodgkin
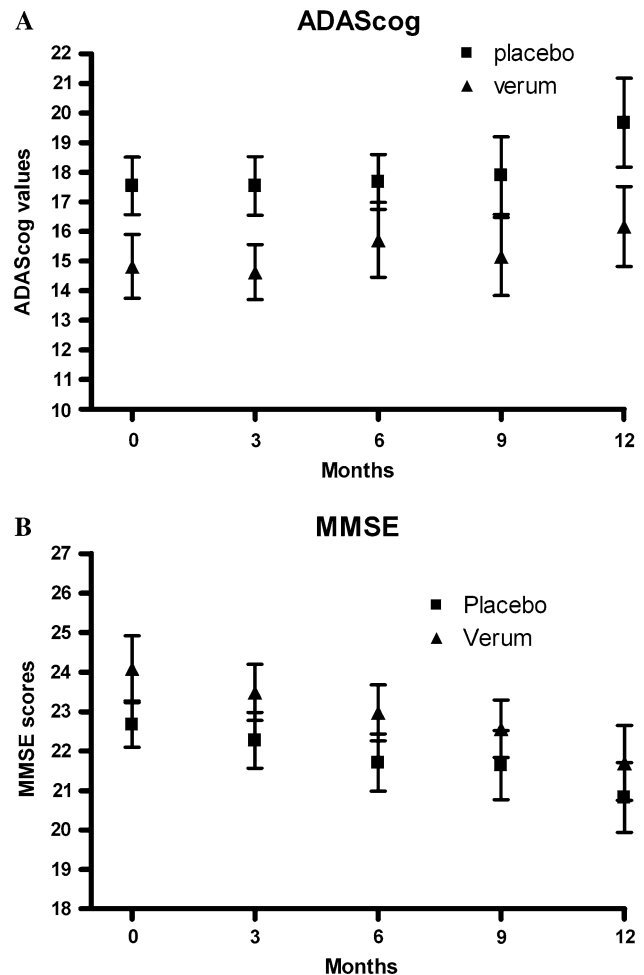

Fig. 2 Mean and standard deviation change in cognitive abilities. **a** Cognitive abilities assessed with ADAS-cog in Cu (verum) vs. placebo arms. **b** Cognitive abilities assessed with MMSE score in Cu (verum) vs. placebo arms

lymphoma (1 patient) and suspected cerebral infarction (1 patient). None of the serious adverse events leading to discontinuation of the study was considered to be attributable to Cu supplementation. Five patients discontinued the participation in the placebo group. No serious adverse events leading to discontinuation were recorded in the placebo group. Three patients withdrew their consent, one failed to return and one discontinued for other reasons. The liver status and hematological characteristics of the patients was monitored at all visits. No significant changes were detected at any time as an effect of Cu treatment.

Altogether, the study medication was well-tolerated. There were no obvious overall trends or clinically relevant differences between the two groups in vital signs, physical examination, hematological characteristics or biochemical analyses.

Discussion

There is increasing evidence that the metalloproteins APP and $A\beta$ are both involved in brain Cu homeostasis. Maynard et al. (2002) have shown that overexpression of the carboxy-terminal fragment of APP elicits significantly reduced Cu levels in transgenic mouse brain. In addition, overexpression of APP in three different lines of APP transgenic mice resulted in significantly reduced Cu levels (Bayer et al. 2003; Maynard et al. 2002; Phinney et al. 2003). This effect might be further enhanced by the reduced bioavailability of Cu due to the accumulation of high amounts of Cu in plaques (Lovell et al. 1998). The APP mediated Cu efflux activity observed in APP overexpressing living cells may also explain Cu deficiency and a subsequently reduced SOD-1 activity. Remarkably, the TxJ mutation of the toxic milk mouse (mutation in the Wilson's disease gene) with a genetically 1.5-fold upregulated Cu level was associated with an increased survival of APP transgenic mice and lowered endogenous murine $A\beta$ levels prior to detectable $A\beta$ plaques formed by the human APP transgene. The CuATPase7b transporter is a P-type ATPase associated with the trans Golgi network. These observations were the basis to hypothesize that restoring brain Cu homeostasis might have a beneficial influence on the progression of AD.

In the present clinical trial, we demonstrate that oral intake of Cu orotate (8 mg Cu daily for 12 months) has no effect on cognitive abilities of AD patients and is neither detrimental nor beneficial. The treatment was generally well-tolerated. The plasma Cu levels declined in the placebo group during the 12 months-period. Such a decline was not observed in the Cu-treated group. Although the placebo group had higher Cu levels as compared to the verum group at the beginning of the study this effect is of interest. However, this observation should be taken with caution, because the statistical significance is rather weak. The analysis of CSF Cu showed that the Cu levels were stabilized only in the Cu-treated group. This observation indicates that oral Cu intake can modulate CSF Cu levels and antagonizes the age-related increase of Cu levels hypothetically by activating the homeostatic system, i.e., balancing the uptake and excretion pathways (H. Kessler et al., in preparation).

These observations are in good agreement with significantly lower levels of blood Cu in those AD patients, who fulfilled the criteria of CSF diagnosis for AD, i.e., at least two of the three markers ($A\beta42$, Tau, Phospho-Tau) were out of the reference range (Kessler et al. 2006). In addition, we demonstrated reduced Cu levels in blood in patients with higher ADAS-cog scores (making more mistakes in this neuropsychological test) (Pajonk et al. 2005), i.e., AD progression is associated with lower Cu levels in blood.

One animal study showed that treatment of 21-months-old Tg2576 mice with clioquinol, a Cu–Zn chelator, inhibited plaque formation and concomitantly increased soluble brain Cu and Zn levels (Cherny et al. 2001). This increase of Cu- and Zn-ions might either be attributed to an inefficiency of the chelator with its known weak affinities for Zn (K1 = 7.0) and for Cu (K1 = 8.9) or even more likely, due to a facilitated uptake in brain of clioquinol–Cu complexes. The latter hypothesis has been experimentally confirmed in vitro (Treiber et al. 2004). Moreover, lowered insoluble $A\beta$ levels (by 49%) and increased soluble $A\beta$ levels (by 50%) were accompanied with elevated Cu levels (and Zn).

When AD patients were treated with clioquinol, the placebo group deteriorated faster than the clioquinol group suggesting a beneficial effect upon clioquinol treatment (Ritchie et al. 2003). This could be due to clioquinol mediating Cu uptake into the cell or the metal-protein-attenuating-compunds properties. In APP transgenic mice Cu supplementation abrogated this clioquinol effect and rescued premature death (Schafer et al. 2007).

Postmortem Cu levels in CNS of AD patients were found to be decreased (Deibel et al. 1996) or unchanged (Loeffler et al. 1996). Controversial results have also been published on the level of Cu in plasma, plasma and brain in AD patients. Plasma Cu levels being within the normal range in AD patients is in good agreement with an earlier study (Jeandel et al. 1989). However, other studies suggest that elevated Cu levels are a risk factor for AD (Squitti et al. 2002, 2003, 2004).

Short-term high Cu intake has been reported not to affect Cu status or functions related to Cu status, only long-term high Cu intake can result in increases in some parameters in young men (Turnlund et al. 2004). Our study is in good agreement with this report adding further

evidence that long-term intake of Cu stabilizes Cu levels in plasma.

The present clinical trial demonstrates that (1) long-term oral intake of 8 mg Cu can be excluded as a risk factor for AD. (2) Long-term oral intake of Cu is well-tolerated by AD patients. (3) Cu intake has no effect on the progression of AD.

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