Decreased CSF levels of L-carnitine in non-apolipoprotein E4 carriers at early stages of Alzheimer's disease.

María Lodeiro^{a*}, Clara Ibáñez^b, Alejandro Cifuentes^b, Carolina Simó^b and Ángel Cedazo-Mínguez^{a*}

^a Department of Neurobiology, Care Sciences and Society, Karolinska Institutet-Alzheimer's Disease Research Center, NOVUM, 5th floor, SE-14186 Stockholm, Sweden.

^b Laboratory of Foodomics, Institute of Food Science Research (CIAL), CSIC. Nicolás Cabrera 9, 28049 Madrid, Spain.

Running title: CSF L-carnitine levels along AD progression.

*Correspondence to:

María Lodeiro and Ángel Cedazo-Mínguez,

Department of Neurobiology, Care Sciences and Society.

Karolinska Institutet-Alzheimer's Disease Research Center.

NOVUM, 5th floor. SE-14186 Stockholm, Sweden.

Phone: +46 8 585 83751.

Fax: +46 8 585 83880.

E-mails: maria.lodeiro@ki.se (María Lodeiro), Angel.Cedazo-Minguez@ki.se (Ángel Cedazo-Mínguez).

ABSTRACT

Increasing evidences suggest that Alzheimer's disease (AD) is a heterogeneous disorder that includes several subtypes with different aetiology and progression. Cerebrospinal fluid (CSF) is being used to find new biomarkers reflecting the complexity of the disease's pathological pathways. We used CSF and clinical data from patients to investigate the status of asymmetric dimethyl-L-arginine, creatine, suberylglycine and L-carnitine along AD progression. These molecules play important roles on mitochondrial function and dysfunctions in mitochondrial metabolism are involved in AD pathology. We found that non-apolipoprotein E4 carriers show lower levels of L-carnitine in CSF early in AD. L-carnitine levels correlate with $A\beta_{42}$ levels and MMSE score, but do not add specificity or sensitivity to the classical AD CSF biomarkers, $A\beta_{42}$, phospho-Tau and total-Tau. Our results suggest apoE genotype-depending differences in L-carnitine synthesis or metabolism along AD, and insinuate that L-carnitine treatments would be more beneficial for AD patients not carrying the apoE4 isoform.

Key words: ApoE4; L-carnitine; biomarkers; CSF; Alzheimer's disease.

INTRODUCTION

Alzheimer's disease (AD) is a chronic progressive neurodegenerative disorder characterized by cognitive and psychiatric symptoms, being the main cause of dementia [1]. AD currently affects nearly 2% of the population in industrialized countries and the risk dramatically increases in individuals beyond the age of 70; indeed, it is diagnosed to more than 50% of people above the age of 85 [2, 3]. It is predicted that the incidence of AD will triple within the next 50 years [4] which, among other things, supposes a high economic burden. Increasing evidences suggest that AD is a heterogeneous and multifactorial disorder resulting from interactions between the genetic susceptibility and environmental factors [5]. The most important genetic susceptibility factor is the presence of the e4 allele of apolipoprotein E (apoE) that accounts for approximately half of late onset AD [6].

The lack of homogeneity would imply the existence of several subtypes with different aetiology and progression. This concept has not been properly investigated, although it could be of great importance for a proper diagnosis, evaluation of disease progression and adjusting therapeutics strategies. During recent years, a great effort has been made in improving early diagnosis and in disease modifying treatments for AD. However, until now, all the therapeutical approaches based on regulating or eliminating A β from the brain have failed [7], perhaps due to variations in the aetiology of the disease among the patients on the trials.

Cerebrospinal fluid (CSF) represents an important source for the searching and discovering of new biomarkers, as its composition is directly related to metabolite production in the brain [8, 9]. Up to date, three biomarkers in CSF (total Tau protein, A β protein and phosphorylated Tau protein) fulfil the criteria required by the consensus group [10]. The ratio between T-tau and A β_{42} levels has been proposed as the most sensitive for diagnostic purposes [11]. However, the sensitivity and specificity of these biomarkers permit the discrimination between controls and AD patients in late stages, but it is still poor in early stages of the disease. Thus, novel, and

specifically early biomarkers, will aid in diagnosis, monitor disease progression and response to treatment.

We recently used a non-targeted metabolomic approach to analyze differences in the metabolic pattern of CSF samples from subjects with different cognitive status related to AD progression. We found a high prediction power (98.7% of accuracy and specificity and sensitivity values above of 95%) using a combination of novel metabolites and classical AD biomarkers [12, 13]. Abnormalities in cerebral metabolism have been documented widely over the past 50 years in neurodegenerative diseases, including AD, with decreased cerebral metabolism preceding clinical or neuroanatomical development of the disease [14]. Thus, some of these metabolites contributing to an enhanced diagnostic power of AD may reflect early abnormalities in the cerebral metabolism leading to disease progression. Of particular interest could be those ones reflecting dysfunctions in mitochondrial metabolism, since it is believed that mitochondrial alterations are involved in loss of cognitive function, as well as in the exacerbation of structural changes in the pattern of AD pathology [14]. From the group of metabolites contributing to an enhanced diagnostic power [12, 13], here we focused in investigating those reflecting mitochondrial alterations, namely asymmetric dimethyl-L-arginine (ADMA), creatine, suberylglycine and L-carnitine [15-18]. We used CSF and clinical data from patients with mild AD, subjective cognitive impairment (SCI), and mild cognitive impairment (MCI), a state considered of high risk for AD. We analysed if levels of these metabolites were abnormal in AD or MCI and their possible association with AD hallmarks, apoE genotype, gender and mini mental score (MMSE). We report that the levels of L-carnitine were significantly decreased in the CSF of MCI and AD patients that were non-apoE4 carriers, showing positive correlations with CSF A β ₄₂ levels and MMSE score. The implications of these findings for mitochondrial functioning, early diagnosis and L-carnitine supplementation treatment approaches in AD are discussed.

MATERIAL AND METHODS

Study population

The patients included in the study (n=73) were from the Memory Clinic at the Karolinska University Hospital in Huddinge, Sweden: 19 had subjective cognitive impairment (SCI), 31 mild cognitive impairment (MCI) and 23 mild AD. These patients were all living independently in the community. They were evaluated according to a standard comprehensive assessment protocol including clinical examination, brain imaging, electroencephalography, analysis of blood and CSF (including total Tau (T-tau), phospho-Tau (P-tau) and A β ₄₂) and a detailed neuropsychological evaluation. Dementia and AD were diagnosed according to DSM-IV and NINCDS-ADRDA criteria. MCI patients were (1) not demented, (2) had (self and/or an informant) reported cognitive decline and impairment on objective cognitive tasks, and (3) had preserved basic ADL/minimal impairment in complex instrumental functions. SCI patients had cognitive complaints without impairment on objective cognitive tasks. Women under hormonal replacement therapy, as well as patients with psychiatric disorders (i.e., depression, alcohol abuse) or other conditions (i.e., diabetes, brain tumors, normal pressure hydrocephalus) were not included. The study was conducted under the guidelines of the Declaration of Helsinki and approved by the ethics committee of the Karolinska Institutet.

CSF measurements

CSF was collected for diagnostic purpose by lumbar puncture as previously described [19, 20]. CSF samples were obtained by lumbar puncture performed in the sitting position. CSF extraction is routinely performed at the Karolinska University Hospital Memory Clinic in Huddinge (Sweden) as part of the medical examination. The extractions were performed in the mornings in fasting patients. CSF samples were obtained from L3/L4 or L4/L5 interspaces after local anesthetic infiltration on the skin. After disposal of the first ml. the following 10 ml were collected in polypropylene tubes. No sample containing more than 500 erythrocytes/ μ l CSF samples was used. Samples were gently mixed to avoid gradient effects and centrifuged at

2000xg for 10 minutes to eliminate cells and insoluble material. Supernatants were aliquoted, immediately frozen and stored at -80°C pending biochemical analysis. Total-Tau was determined using a sandwich enzyme-linked immunoabsorbent assay (ELISA) [21]. P-Tau (P-Thr 181) was determined using a sandwich ELISA with monoclonal antibody HT7 (recognizing all forms of Tau) used as capturing antibody, and AT270 (specific to P(Thr181)-Tau used as a detection antibody. [22] A β ₄₂ was determined using a sandwich ELISA as previously described[19]. All kits were purchased from Innogenetics NV, Ghent, Belgium.

Metabolite levels were measured using a new metabolomic approach based on capillary electrophoresis-mass spectrometry (CE-MS) [12]. CE analyses were carried out in a P/ACE 5500 CE apparatus from Beckman Instruments (Fullerton, CA, USA). The instrument was controlled by a PC running the System Gold software from Beckman. Uncoated fused-silica capillaries (50 μ m id and 87 cm total length) from Composite Metal Services (Worcester, England) were coupled to the mass spectrometer through an orthogonal electrospray ionization (ESI) interface model G1607A from Agilent Technologies (Palo Alto, CA, USA). Electrical contact at the electrospray needle tip was established via a sheath liquid. A time of flight (TOF) MS instrument (micrOTOF) from Bruker Daltonics (Bremen, Germany) was employed. The instrument was controlled by a PC running the micrOTOF control software from Bruker Daltonics. Injections were made at the anodic end using N₂ pressure of 0.5 psi (34.5 mbar) for 80 s. The electrophoretic separation was achieved applying +25 kV as running voltage at a constant temperature of 25 °C. The micrOTOF was operated to acquire spectra in the m/z range of 50-500 [12].

Data analysis

Normal distribution of protein data was checked by Shapiro-Wilks. Metabolites are presented as mean values $\pm$ standard error mean (SEM). CSF metabolite levels along the different groups were analyzed using ANOVA. Pairwise comparisons were performed by HSD Tukey's post-hoc test. For interaction analysis, two-way ANOVA followed by Bonferroni post-hoc test was used.

Associations between A β ₄₂, Total-Tau or P-tau levels, MMSE score, apoE genotype and CSF carnitine levels were assessed by partial correlation analysis. Significance was defined as $p \leq 0.05$.

CE-MS metabolite data was processed by using the open-source bioinformatics tool MZmine [12]. Briefly, this software was applied to the experimental information obtained by the mass spectrometer to obtain a final data matrix containing the relative metabolite concentration (related to MS peak areas) from each metabolite found in all the CSF samples. The relative concentrations of metabolites were compared among samples by statistical analysis and finally, a statistical validation was assessed by a leave-one-out cross-validation procedure [12].

RESULTS

General characteristics of the population

Demographic and clinical characteristics of study participants are shown in Table 1. Mild AD patients were significantly older than SCI and MCI ($F(2,70) = 13.95$, $p < 0.001$). MMSE score was lower in mild AD patients compared to SCI and MCI patients ($F(2,70) = 32.33$, $p < 0.001$).

Mild AD patients presented lower CSF levels of $A\beta_{42}$ and higher T-tau and P-tau when compared to either SCI ($F(2,66) = 13.29$, $p < 0.001$; $F(2,69) = 10.83$, $p < 0.001$; $F(2,58) = 9.02$, $p < 0.01$) or MCI ($p < 0.05$; $p < 0.001$; $p < 0.01$, respectively).

L-Carnitine levels are decreased in CSF of AD patients and correlate with CSF $A\beta_{42}$ levels and MMSE score.

Analysis of L-carnitine, suberylglycine, ADMA and creatine in CSF were performed and are shown in Figure 1A. No significant differences were found between groups in the levels of suberylglycine, ADMA or creatine. However, we found significant reductions of L-carnitine levels in CSF of patients with mild AD (0.0191 ± 0.0021) and MCI (0.0196 ± 0.0016) compared to SCI (0.0265 ± 0.0016) (one way ANOVA followed by HSD Tukey's test, $F(2,70) = 4.42$, $p = 0.0155$ and $p < 0.05$, respectively; Figure 1A).

CSF levels of L-carnitine correlated directly with $A\beta_{42}$ levels ($r = 0.2875$, $n = 69$, $p = 0.0166$) and MMSE score ($r = 0.3130$, $n = 73$, $p = 0.0070$) (Figure 1B). However, no correlation between L-carnitine levels and T-Tau ($r = -0.2127$, $n = 72$, $p = 0.0728$), P-Tau ($r = -0.2121$, $n = 61$, $p = 0.1007$) or age ($r = -0.08598$, $n = 73$, $p = 0.4695$) were found.

Decreased CSF L-carnitine levels in non-apoE4 carriers patients.

Figure 2A shows the gender-stratified distribution of CSF-L-carnitine levels along the three different groups. On average, women and men presented similar levels (0.02135 ± 0.0015 versus 0.02104 ± 0.0015 a.u.; $p=0.88$). There was no interaction between groups and gender.

Average levels of CSF L-carnitine between ApoE4 carriers and non-carriers did not differ (0.0210 ± 0.0016 versus 0.0212 ± 0.0013 a.u.; $p = 0.91$. Figure 2B). An interaction between groups and genotype was found ($F(2,64) = 3.64$; $p = 0.0318$ two-way ANOVA followed by Bonferroni post-hoc test). A group effect was also shown, and differences were found among non-E4 carriers in the SCI compare to the mild-AD ($F(2,64) = 3.70$; $p = 0.0301$. Figure 2B).

When the $A\beta_{42}$ and MMSE correlations were done taking into account the apoE4 genotype, we found only significant correlations in the group of non-E4 carriers (Figure 2C and D) for both $A\beta_{42}$ ($r = 0.4979$, $n = 35$, $p = 0.0023$) and MMSE score ($r = 0.5162$, $n = 36$, $p = 0.0013$), but no for apoE4 carriers (Figures 2E and F).

L-carnitine did not improve the diagnostic power of classical AD biomarkers in non-apoE4 carriers.

Numerous studies have explored the use of the CSF concentration of classical AD hallmarks as biomarkers for an early diagnosis. The ratio between T-tau and $A\beta_{42}$ levels has been proposed as the most sensitive for diagnostic proposes [23]. To explore the possibility of using L-carnitine values in combination with T-tau and $A\beta_{42}$ for improving diagnose accuracy in non-apoE4 carriers, we calculated the area under the curve (AUC) for the T-Tau/ ($A\beta_{42} * L\text{-carnitine}$). In all patients of the study, the AUC for the T-Tau/ $A\beta_{42}$ ratio was 0.86 . This was not improved by L-carnitine (AUC= 0.87; Figure 3A). When we performed the same analysis only among the apoE4 non-carriers patients, the AUC values changed from 0.80 to 0.89 respectively (Figure 3B). This change was not statistically significant ($p=0.19$).

DISCUSSION

Since it is suggested that AD is a heterogeneous disorder that includes several aetiologies, a better characterization of causes and subtypes would help to improve diagnosis and treatment efficiency. Several other reports indicate differences in AD biomarkers, progression or pathogenesis related to gender or to the apoE genotype [24]. For example, we previously showed that levels of insulin in CSF are decreased among women, but no men, in prodromal AD. This decrease was associated with lower A β ₄₂ levels and cognitive score [25].

From the complexity of AD pathology, there is an increasing consensus pointing that disruption of mitochondrial function could be an early event in the pathological cascade [15, 26]. Mitochondria are highly dynamic organelles that not only supply the main energy to the cell but also regulate apoptosis [27]. The changes suffered by mitochondria during AD pathology include: morphology, increase in permeability of mitochondrial membranes, reduction of mass, decrease in the activities of enzymes, increase in mtDNA fragmentation, deregulation in fission/fusion processes and transport, alteration of oxidative stress with a overproduction of ROS and a deregulation of apoptosis. Therefore, decline in mitochondrial function impacts many cellular processes, such as inflammation, autophagy, and apoptosis, needed for the proper maintenance of the cellular function [26, 28].

We recently showed changes in the metabolite pattern in CSF during the progression of AD that could help to an early diagnosis of the disease. Four of them, ADMA, creatine, suberylglycine and L-carnitine, have well reported roles in mitochondrial function [15-18].

Here, we report that levels of L-carnitine in CSF were decreased in patients in the MCI and mild AD groups compared to SCI. Moreover, CSF L-carnitine levels directly correlated to CSF A β ₄₂ levels and MMSE score, but not to the other classical AD biomarkers (T-Tau, P-Tau). On average, women and men presented similar levels, but when we stratified the study population for the apoE genotype, we found that decreased CSF L-carnitine was seen only in non-apoE4 carriers, with more pronounced correlations with A β ₄₂ levels and MMSE score. Despite these

differences, not improvements in either sensitivity or specificity were found when combining L-carnitine CSF measurements with the classical AD biomarkers (T-tau/A β ₄₂ ratio) both in the general population and in the non-apoE4 carriers.

Carnitine-acetyl-O transferase converts L-carnitine and acetyl-Coenzyme A (acetyl-CoA) in acetyl-L-carnitine (ALC) and CoA in the mitochondria. ALC is transported also outside of the mitochondria where it is converted back to its precursors. This process i) facilitates the uptake of acetyl CoA into the mitochondria during fatty acid oxidation, ii) enhances the acetylcholine production and iii) stimulates protein and membrane phospholipid synthesis. The mechanism of action of ALC includes enhance neuronal metabolism in the mitochondria, ability to stabilize cell membrane fluidity, and prevention of cell death and neuronal damage. In addition, it also promotes an increase in the hippocampal binding of glucocorticoids and nerve growth factor and reduces the oxidative stress. [29].

In a previous study, no significant differences were found in free carnitine (FC) and acyl-carnitine esters between CSF samples from patients with AD and controls and they did not correlate with age, age at onset of AD, duration of AD or scores in the MMSE [30]. On the other hand the beneficial effects of ALC in preventing the loss of age-related brain function and neurodegenerative disorders have been widely studied [31-34]. Regarding AD, it has been described that ALC treatment improves cognitive performance in patients [35]. Several reports showed positive effects *in vitro* of ALC on A β toxicity, through the prevention of A β -induced mitochondrial damage and ATP depletion [36]; on A β deposition, through the up-regulation of kinesin light-chain 1, a protein involved in the fast anterograde axonal transport of A β peptide [37]; and on A β production, through stimulation of α -secretase activity (ADAM 10) [38]. Besides, ALC has also many neuromodulatory and neurotrophic actions on neuronal activity helping to maintain mitochondrial bioenergetics and lowering oxidative stress associated with aging [31, 32]. An increasing number of studies suggest that other mechanisms than the antioxidant activities could be involved in the neuroprotective effect of carnitine such as the activation of heat-shock proteins, which can exert neuroprotective effects against oxidative

stress-related injury [31, 39]. In animal models, L-carnitine was shown to reverse the homocysteine- induced tau hyperphosphorylation, A β accumulation and memory deficits in rats [40]. Besides, L-carnitine supplementation was shown to improve cognition and to reduce oxidative and mitochondrial damage in the brain of aged rats [33, 41]. These evidences support the possibility that L-carnitine may provide therapeutic benefits in oxidative stress-associated neurodegenerative diseases such as AD.

Our study brings evidence for a different metabolism of L-carnitine in AD brain, depending upon the apoE genotype. The presence of apoE4 is the most prevalent risk factor for late onset or sporadic AD and some of the pathological features of AD, such as impairment of energy metabolism and mitochondrial dysfunctions are seen earlier in apoE4 homozygotic patients [42]. In a transgenic mice model for human apoE4, it was reported that acetyl-L-carnitine treatment eliminated the neuronal mitochondrial damage and also prevented the formation of lipofuscin and/or myelin-like structures in neurons. Moreover, an improvement of the cognitive function was also observed [34].

However, the presence of apoE4 has been also associated with increased risk for atherosclerosis and for coronary artery disease [43], diseases where some negative effects of carnitine have been also reported. A recent paper has shown that the metabolism of carnitine, through a specific gut microbiota, produces an intermediate metabolite which accelerates atherosclerosis in mice [44]. Other authors have reported that in ApoE/LDLR^{-/-} mice, treatment with mildronate, an inhibitor of L-carnitine biosynthesis and transport that is used as a cardioprotective drug, decreased the size of atherosclerotic plaques and L-carnitine concentrations of aortic tissues [45]. A report from the carotid ultrasound disease assessment study (CUDAS), found a significant association between apoE4 and increased incidence of carotid plaque but not of intima-media thickening (IMT)[46]. However, Altamura et al., have reported an increase of the IMT values in AD patients carrying the apoE4 allele respect to non-apoE4 AD-carriers, proposing that apoE4 might promote IMT interacting with other factors contributing to AD development [47].

The efficacy of ALC in cognitive decline or AD has been investigated in several clinical trials, yet with unclear results. Cochrane reviewed in 2003 of 11 ALC clinical trials in AD showed benefits of ALC in clinical global impression but not in cognition [48]. In recent years, still conflicting results have been reported [35, 49-52]. To our knowledge, these studies were never stratified for the apoE genotype. In view of our results, where AD patients carrying apoE4 do not appear to have deficits in brain ALC, while non-apoE4 carriers do, and considering that both, increased ALC and the presence of apoE4, increase the risk for atherosclerosis, we propose that dietary L-carnitine supplementation will be more beneficial for non-apoE4 carriers with AD even at MCI stages.

In summary, we report that during the progression of AD, non-apoE4 carriers show lower levels of L-carnitine in CSF. However, this difference does not add specificity or sensitivity to the classical AD CSF biomarkers, T-Tau/A β ₄₂ ratio. The mechanisms behind the apoE isoform differences on L-carnitine synthesis or metabolism in brain should be investigated. Since both, high L-carnitine levels and apoE4 genotype confer higher risk for atherosclerosis and for coronary artery disease, our results suggest that L-carnitine treatments would be more beneficial for AD patients do not carrying the apoE4 isoform.

ACKNOWLEDGEMENTS

This research was supported by grants from Sweden: Swedish Brain Power, Alzheimerfonden, Gun och Bertil Stohnes Stiftelse, Karolinska Institutet fund for geriatric research, Stiftelsen Gamla Tjänarinnor, Stiftelsen Lars Hiertas Minne, Stiftelsen Dementia, Demensförbundet and the regional agreement on medical training and clinical research (ALF) between Stockholm County Council and the Karolinska Institute.

Disclosure statement

The authors report no conflict of interest including financial or personal.

REFERENCES

- [1] Blennow K, de Leon MJ, Zetterberg H (2006) Alzheimer's disease. *Lancet* **368**, 387-403.
- [2] Golomb J, Kluger A, Ferris SH (2004) Mild cognitive impairment: historical development and summary of research. *Dialogues Clin Neurosci* **6**, 351-367.
- [3] Mattson MP (2004) Pathways towards and away from Alzheimer's disease. *Nature* **430**, 631-639.
- [4] Wimo A, Reed CC, Dodel R, Belger M, Jones RW, Happich M, Argimon JM, Bruno G, Novick D, Vellas B, Haro JM (2013) The GERAS Study: a prospective observational study of costs and resource use in community dwellers with Alzheimer's disease in three European countries--study design and baseline findings. *Journal of Alzheimers Disease* **36**, 385-399.
- [5] Hardy J, Selkoe DJ (2002) The amyloid hypothesis of Alzheimer's disease: progress and problems on the road to therapeutics. *Science* **297**, 353-356.
- [6] Cedazo-Minguez A (2007) Apolipoprotein E and Alzheimer's disease: molecular mechanisms and therapeutic opportunities. *J Cell Mol Med* **11**, 1227-1238.
- [7] Mangialasche F, Solomon A, Winblad B, Mecocci P, Kivipelto M (2010) Alzheimer's disease: clinical trials and drug development. *Lancet Neurol* **9**, 702-716.
- [8] Hampel H, Prvulovic D, Teipel S, Jessen F, Luckhaus C, Frolich L, Riepe MW, Dodel R, Leyhe T, Bertram L, Hoffmann W, Faltraco F (2011) The future of Alzheimer's disease: the next 10 years. *Prog Neurobiol* **95**, 718-728.
- [9] Johanson CE, Duncan JA, 3rd, Klinge PM, Brinker T, Stopa EG, Silverberg GD (2008) Multiplicity of cerebrospinal fluid functions: New challenges in health and disease. *Cerebrospinal Fluid Res* **5**, 10.
- [10] Mattsson N, Zegers I, Andreasson U, Bjerke M, Blankenstein MA, Bowser R, Carrillo MC, Gobom J, Heath T, Jenkins R, Jeromin A, Kaplow J, Kidd D, Laterza OF, Lockhart A, Lunn MP, Martone RL, Mills K, Pannee J, Ratcliffe M, Shaw LM, Simon

- AJ, Soares H, Teunissen CE, Verbeek MM, Umek RM, Vanderstichele H, Zetterberg H, Blennow K, Portelius E (2012) Reference measurement procedures for Alzheimer's disease cerebrospinal fluid biomarkers: definitions and approaches with focus on amyloid beta42. *Biomark Med* **6**, 409-417.
- [11] Cedazo-Minguez A, Winblad B (2010) Biomarkers for Alzheimer's disease and other forms of dementia: clinical needs, limitations and future aspects. *Exp Gerontol* **45**, 5-14.
- [12] Ibanez C, Simo C, Martin-Alvarez PJ, Kivipelto M, Winblad B, Cedazo-Minguez A, Cifuentes A (2012) Toward a predictive model of Alzheimer's disease progression using capillary electrophoresis-mass spectrometry metabolomics. *Anal Chem* **84**, 8532-8540.
- [13] Ibanez C, Simo C, Barupal DK, Fiehn O, Kivipelto M, Cedazo-Minguez A, Cifuentes A (2013) A new metabolomic workflow for early detection of Alzheimer's disease. *J Chromatogr A* **1302**, 65-71.
- [14] Salek RM, Xia J, Innes A, Sweatman BC, Adalbert R, Randle S, McGowan E, Emson PC, Griffin JL (2010) A metabolomic study of the CRND8 transgenic mouse model of Alzheimer's disease. *Neurochem Int* **56**, 937-947.
- [15] Kidd PM (2005) Neurodegeneration from mitochondrial insufficiency: nutrients, stem cells, growth factors, and prospects for brain rebuilding using integrative management. *Altern Med Rev* **10**, 268-293.
- [16] Selley ML (2003) Increased concentrations of homocysteine and asymmetric dimethylarginine and decreased concentrations of nitric oxide in the plasma of patients with Alzheimer's disease. *Neurobiol Aging* **24**, 903-907.
- [17] Klopstock T, Elstner M, Bender A (2011) Creatine in mouse models of neurodegeneration and aging. *Amino Acids* **40**, 1297-1303.
- [18] Longo N, Schrijver I, Vogel H, Pique LM, Cowan TM, Pasquali M, Steinberg GK, Hedlund GL, Ernst SL, Gallagher RC, Enns GM (2008) Progressive cerebral vascular degeneration with mitochondrial encephalopathy. *Am J Med Genet A* **146**, 361-367.

- [19] Hansson O, Zetterberg H, Buchhave P, Londos E, Blennow K, Minthon L (2006) Association between CSF biomarkers and incipient Alzheimer's disease in patients with mild cognitive impairment: a follow-up study. *Lancet Neurol* **5**, 228-234.
- [20] Andreasen N, Hesse C, Davidsson P, Minthon L, Wallin A, Winblad B, Vanderstichele H, Vanmechelen E, Blennow K (1999) Cerebrospinal fluid beta-amyloid(1-42) in Alzheimer disease: differences between early- and late-onset Alzheimer disease and stability during the course of disease. *Arch Neurol* **56**, 673-680.
- [21] Blennow K, Wallin A, Agren H, Spenger C, Siegfried J, Vanmechelen E (1995) Tau protein in cerebrospinal fluid: a biochemical marker for axonal degeneration in Alzheimer disease? *Mol Chem Neuropathol* **26**, 231-245.
- [22] Vanmechelen E, Vanderstichele H, Davidsson P, Van Kerschaver E, Van Der Perre B, Sjogren M, Andreasen N, Blennow K (2000) Quantification of tau phosphorylated at threonine 181 in human cerebrospinal fluid: a sandwich ELISA with a synthetic phosphopeptide for standardization. *Neurosci Lett* **285**, 49-52.
- [23] Cedazo-Minguez A, Winblad B (2010) Biomarkers for Alzheimer's disease and other forms of dementia: clinical needs, limitations and future aspects. *Experimental Gerontology* **45**, 5-14.
- [24] Ashford JW (2004) APOE genotype effects on Alzheimer's disease onset and epidemiology. *J Mol Neurosci* **23**, 157-165.
- [25] Gil-Bea FJ, Solas M, Solomon A, Mugueta C, Winblad B, Kivipelto M, Ramirez MJ, Cedazo-Minguez A (2010) Insulin levels are decreased in the cerebrospinal fluid of women with prodromal Alzheimer's disease. *J Alzheimers Dis* **22**, 405-413.
- [26] Silva DF, Selfridge JE, Lu J, E L, Cardoso SM, Swerdlow RH (2012) Mitochondrial abnormalities in Alzheimer's disease: possible targets for therapeutic intervention. *Adv Pharmacol* **64**, 83-126.
- [27] Verri M, Pastoris O, Dossena M, Aquilani R, Guerriero F, Cuzzoni G, Venturini L, Ricevuti G, Bongiorno AI (2012) Mitochondrial alterations, oxidative stress and

- neuroinflammation in Alzheimer's disease. *Int J Immunopathol Pharmacol* **25**, 345-353.
- [28] Gu XM, Huang HC, Jiang ZF (2012) Mitochondrial dysfunction and cellular metabolic deficiency in Alzheimer's disease. *Neurosci Bull* **28**, 631-640.
- [29] (2010) Acetyl-L-carnitine. Monograph. *Altern Med Rev* **15**, 76-83.
- [30] Rubio JC, de Bustos F, Molina JA, Jimenez-Jimenez FJ, Benito-Leon J, Martin MA, Campos Y, Orti-Pareja M, Cabrera-Valdivia F, Arenas J (1998) Cerebrospinal fluid carnitine levels in patients with Alzheimer's disease. *J Neurol Sci* **155**, 192-195.
- [31] Pettegrew JW, Levine J, McClure RJ (2000) Acetyl-L-carnitine physical-chemical, metabolic, and therapeutic properties: relevance for its mode of action in Alzheimer's disease and geriatric depression. *Mol Psychiatry* **5**, 616-632.
- [32] Virmani A, Binienda Z (2004) Role of carnitine esters in brain neuropathology. *Mol Aspects Med* **25**, 533-549.
- [33] Suchy J, Chan A, Shea TB (2009) Dietary supplementation with a combination of alpha-lipoic acid, acetyl-L-carnitine, glycerophosphocoline, docosahexaenoic acid, and phosphatidylserine reduces oxidative damage to murine brain and improves cognitive performance. *Nutr Res* **29**, 70-74.
- [34] Shenk JC, Liu J, Fischbach K, Xu K, Puchowicz M, Obrenovich ME, Gasimov E, Alvarez LM, Ames BN, Lamanna JC, Aliev G (2009) The effect of acetyl-L-carnitine and R-alpha-lipoic acid treatment in ApoE4 mouse as a model of human Alzheimer's disease. *J Neurol Sci* **283**, 199-206.
- [35] Montgomery SA, Thal LJ, Amrein R (2003) Meta-analysis of double blind randomized controlled clinical trials of acetyl-L-carnitine versus placebo in the treatment of mild cognitive impairment and mild Alzheimer's disease. *Int Clin Psychopharmacol* **18**, 61-71.
- [36] Dhitavat S, Ortiz D, Rogers E, Rivera E, Shea TB (2005) Folate, vitamin E, and acetyl-L-carnitine provide synergistic protection against oxidative stress resulting from exposure of human neuroblastoma cells to amyloid-beta. *Brain Res* **1061**, 114-117.

- [37] Traina G, Federighi G, Brunelli M (2008) Up-regulation of kinesin light-chain 1 gene expression by acetyl-L-carnitine: therapeutic possibility in Alzheimer's disease. *Neurochem Int* **53**, 244-247.
- [38] Epis R, Marcello E, Gardoni F, Longhi A, Calvani M, Iannuccelli M, Cattabeni F, Canonico PL, Di Luca M (2008) Modulatory effect of acetyl-L-carnitine on amyloid precursor protein metabolism in hippocampal neurons. *Eur J Pharmacol* **597**, 51-56.
- [39] Abdul HM, Calabrese V, Calvani M, Butterfield DA (2006) Acetyl-L-carnitine-induced up-regulation of heat shock proteins protects cortical neurons against amyloid-beta peptide 1-42-mediated oxidative stress and neurotoxicity: implications for Alzheimer's disease. *J Neurosci Res* **84**, 398-408.
- [40] Zhou P, Chen Z, Zhao N, Liu D, Guo ZY, Tan L, Hu J, Wang Q, Wang JZ, Zhu LQ (2011) Acetyl-L-carnitine attenuates homocysteine-induced Alzheimer-like histopathological and behavioral abnormalities. *Rejuvenation Res* **14**, 669-679.
- [41] Aliev G, Liu J, Shenk JC, Fischbach K, Pacheco GJ, Chen SG, Obrenovich ME, Ward WF, Richardson AG, Smith MA, Gasimov E, Perry G, Ames BN (2009) Neuronal mitochondrial amelioration by feeding acetyl-L-carnitine and lipoic acid to aged rats. *J Cell Mol Med* **13**, 320-333.
- [42] Reiman EM, Caselli RJ, Chen K, Alexander GE, Bandy D, Frost J (2001) Declining brain activity in cognitively normal apolipoprotein E epsilon 4 heterozygotes: A foundation for using positron emission tomography to efficiently test treatments to prevent Alzheimer's disease. *Proc Natl Acad Sci U S A* **98**, 3334-3339.
- [43] Hofman A, Ott A, Breteler MM, Bots ML, Slioter AJ, van Harskamp F, van Duijn CN, Van Broeckhoven C, Grobbee DE (1997) Atherosclerosis, apolipoprotein E, and prevalence of dementia and Alzheimer's disease in the Rotterdam Study. *Lancet* **349**, 151-154.
- [44] Koeth RA, Wang Z, Levison BS, Buffa JA, Org E, Sheehy BT, Britt EB, Fu X, Wu Y, Li L, Smith JD, DiDonato JA, Chen J, Li H, Wu GD, Lewis JD, Warrier M, Brown JM, Krauss RM, Tang WH, Bushman FD, Lusis AJ, Hazen SL (2013) Intestinal microbiota

- metabolism of L-carnitine, a nutrient in red meat, promotes atherosclerosis. *Nat Med* **19**, 576-585.
- [45] Vilskersts R, Liepinsh E, Mateuszuk L, Grinberga S, Kalvinsh I, Chlopicki S, Dambrova M (2009) Mildronate, a regulator of energy metabolism, reduces atherosclerosis in apoE/LDLR^{-/-} mice. *Pharmacology* **83**, 287-293.
 - [46] Beilby JP, Hunt CC, Palmer LJ, Chapman CM, Burley JP, McQuillan BM, Thompson PL, Hung J (2003) Apolipoprotein E gene polymorphisms are associated with carotid plaque formation but not with intima-media wall thickening: results from the Perth Carotid Ultrasound Disease Assessment Study (CUDAS). *Stroke* **34**, 869-874.
 - [47] Altamura C, Squitti R, Pasqualetti P, Tibuzzi F, Silvestrini M, Ventriglia MC, Cassetta E, Rossini PM, Vernieri F (2007) What is the relationship among atherosclerosis markers, apolipoprotein E polymorphism and dementia? *Eur J Neurol* **14**, 679-682.
 - [48] Hudson S, Tabet N (2003) Acetyl-L-carnitine for dementia. *Cochrane Database Syst Rev*, CD003158.
 - [49] Gavrilova SI, Kalyn Ia B, Kolykhalov IV, Roshchina IF, Selezneva ND (2011) [Acetyl-L-carnitine (carnicetine) in the treatment of early stages of Alzheimer's disease and vascular dementia]. *Zh Nevrol Psikhiatr Im S S Korsakova* **111**, 16-22.
 - [50] Bianchetti A, Rozzini R, Trabucchi M (2003) Effects of acetyl-L-carnitine in Alzheimer's disease patients unresponsive to acetylcholinesterase inhibitors. *Curr Med Res Opin* **19**, 350-353.
 - [51] Thal LJ, Calvani M, Amato A, Carta A (2000) A 1-year controlled trial of acetyl-L-carnitine in early-onset AD. *Neurology* **55**, 805-810.
 - [52] Thal LJ, Carta A, Clarke WR, Ferris SH, Friedland RP, Petersen RC, Pettegrew JW, Pfeiffer E, Raskind MA, Sano M, Tuszynski MH, Woolson RF (1996) A 1-year multicenter placebo-controlled study of acetyl-L-carnitine in patients with Alzheimer's disease. *Neurology* **47**, 705-711.

Tables

Table 1: Demographic and clinical characteristics of study participants.

<i>Demographics</i>	SCI	MCI	Mild-AD
n	19	31	23
Gender (male/female)	(7/12)	(18/13)	(5/18)
Age (years)	57.3 (1.44)	60 (1.6)	70 (1.9)
Education (years)	14.6 (0.7)	12.8 (0.6)	9.3 (0.6)
ApoE carriers (%)	7/19 (36,84%)	16 /31 (51,6%)	11/23 (47,82%)
MMSE (points)	28.9 (0.3)	28 (0.3)	23.2 (0.8)
<i>Clinical data</i>			
Q ALB	6.7 (0.7)	6.5 (0.6)	6.8 (0.5)
CSF A β_{1-42} (ng/L)	830.3 (43.1)	651.2 (43.8)	516.5 (26.8)
CSF T-Tau (ng/L)	290.9 (34.6)	332.3 (34.9)	602.7 (72.3)
CSF P-Tau (ng/L)	50.8 (3.8)	52.9 (5.2)	86.8 (8.2)

Numeric values are presented as number of patients, percentage or mean ($\pm$ SEM).

SCI: subjective cognitive impairment

MCI: mild cognitive impairment

Mild AD: mild Alzheimer's disease

MMSE: Mini-Mental State Examination

CSF: cerebrospinal fluid

Alb: albumin

FIGURE LEGENDS

Figure 1: L-carnitine is decreased in CSF from MCI and AD patients and correlates with A β ₄₂ and MMSE score. Figure 1A shows CSF levels of L-carnitine, suberylglycine, asymmetric dimethyl-L-arginine (ADMA) and creatine. Only L-carnitine determinations showed statistically significant changes along AD progression (* $p < 0.05$, one-way ANOVA, Tukey post-hoc test). Figure 1B shows correlation analysis between CSF levels of L-carnitine and CSF A β ₄₂ levels ($r = 0.2875$, $p = 0.0166$; left figure) and MMSE score ($r = 0.3130$; $p = 0.007$; right figure).

Figure 2: L-carnitine is decreased in CSF of apoE4 non-carriers in AD. Figure 2A shows CSF L-carnitine levels stratified by gender. Non significant static differences were found between men and women. Figure 2B represents CSF L-carnitine levels in apoE4 carriers or non-carriers in the different diagnosed groups. Levels of L-carnitine were significantly lower on apoE4-non carriers with AD (* $p = 0.0301$; two-way ANOVA followed by Bonferroni post-hoc analysis). Figure 2C and 2D shows correlations of CSF L-carnitine and CSF A β ₄₂ levels (Fig.C; $r = 0.4979$, $p = 0.0023$) or MMSE score (Fig.D; $r = 0.5162$, $p = 0.0013$). In apoE4 carriers, no correlations were found between CSF L-carnitine and CSF A β ₄₂ levels (Fig.2E) or MMSE score (Fig.2F).

Figure 3: L-carnitine does not improve the sensitivity and specificity of classical AD biomarkers. The plot shows a ROC curve analysis for all patients from the groups of the study (MCI and mild AD) versus controls (SCI). In the general population of the study, the AUC was 0.86 for T-Tau/A β ₄₂ ratio (Fig.3A, red line) and 0.87 for T-Tau/(A β ₄₂*L-carnitine) (Fig.3A, black line). Figure 3B shows the same analysis for apoE4 non carriers; the AUC was 0.80 for T-Tau/A β ₄₂ ratio (Fig.3B, red line) and 0.89 for T-Tau/(A β ₄₂*L-carnitine) (Fig.3B, black line). L-carnitine did not improved significantly the diagnostic power of the T-Tau/A β ₄₂ ratio ($p = 0.19$).

Figure 1

A

B

Figure 2

Figure 3