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Correlations between Alzheimer's CSF biomarkers and cerebral glucose metabolism after 12 months of phenserine treatment

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Running title: 12 months phenserine treatment in AD patients.

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Abstract

New therapeutic strategies in Alzheimer´s disease AD) are focused on targeting beta-amyloid (Aβ) to modify the underlying cause of the disease rather than just the symptoms. The aim of this study was to investigate the long-term effects of treatment with the anti-Aβ compound phenserine on (i) cerebrospinal fluid (CSF) biomarkers for Aβ and tau pathology and (ii) brain metabolism as assessed by the regional cerebral metabolic rate for glucose (rCMRglc), using positron emission tomography. Twenty patients with mild AD were included in the study and after 12 months treatment with phenserine CSF Aβ40 and α- and β-secretase-cleaved soluble amyloid precursor protein (sAPP) levels had significantly increased and rCMRglc had stabilized. Levels of CSF Aβ40 and sAPP correlated positively with rCMRglc and cognition while CSF Aβ42 levels, the Aβ42/40 ratio, P-tau and T-tau correlated negatively with rCMRglc and cognition. In summary, long-term phenserine treatment resulted in increased levels of CSF Aβ40, α−sAPP and β-sAPP, which positively correlated with improvements in rCMRglc and cognition. The study illustrates the value of using biomarkers in the CSF and brain for evaluation of drug effects.

Keywords: Alzheimer's disease, Positron emission tomography, Phenserine, Cerebral glucose metabolism, Cerebrospinal fluid.

Introduction

Current therapies for Alzheimer's disease (AD) provide symptomatic relief by either improving symptoms or delaying decline [1]. Increasing knowledge about the molecular mechanisms of AD has provided multiple potential targets for disease-modifying therapeutic intervention. Beta-amyloid (Aβ) plaque accumulation is considered to be an early-stage event central to the neuropathology of AD [2, 3]. The Aβ protein, a 40- to 42-amino acid protein, is generated by proteolytic cleavage from the Aβ precursor protein (APP) by β- and γ-secretase [4]. Aggregated Aβ contained in plaques seems relatively inert but different forms of soluble Aβ oligomers may be toxic to neurons [5-8]. It is generally assumed that therapeutic strategies that target Aβ would modify the underlying causes of the disease rather than just the symptoms.

Several potentially disease-modifying therapies, including both passive and active immunotherapy aiming to promote antibody-mediated clearance of Aβ, are under development for AD [9-12]. Other anti-Aβ approaches focus on inhibition of the γ- and β-secretase enzymes or enhancement of α-secretase activity to decrease Aβ production, and inhibition of Aβ aggregation to prevent the formation of oligomers and Aβ plaques [9, 11, 12,13].

In experimental studies, (–)-phenserine has been shown to have dual modes of action; it acts as both an acetylcholinesterase inhibitor (AChEI) and an inhibitor of APP synthesis [14]. Phenserine interferes with the 5′ untranslated region of human APP mRNA, decreasing the amount of translated APP. Both (–)- and cholinergically inert (+)-phenserine decrease Aβ levels in cell cultures and mouse brain [15], and in Alzheimer Tg2576 mice [16](Lilja et al. 2013). Additionally, (+)-phenserine increases synaptophysin levels in younger mice and cell proliferation in both younger and older mice [16], and may also stimulate neurogenesis [17].

The cognitive scales commonly employed to investigate drug efficacy in this area are relatively insensitive to change. Various biomarkers, including imaging and biochemical biomarkers in cerebrospinal fluid (CSF) and blood, have consequently been proposed as possible surrogate endpoints to demonstrate treatment effects [18].

Positron emission tomography (PET) has been used successfully for measuring functional activity, neuropathology and neurotransmitter changes in patients with AD (Nordberg et al. 2010). ^{18}F-fluorodeoxyglucose (^{18}F-FDG) PET has demonstrated the presence of regional deficits in the cerebral metabolic rate of glucose (rCMRglc) in AD patients, which have been interpreted as impairments in synaptic activity [19]. These metabolic deficits become more noticeable as the disease progresses [20, 21]. ^{18}F-FDG has been used to track the effects of anti-dementia treatments on rCMRglc [22]. The retention of PET Aβ imaging tracer ^{11}C-Pittsburgh compound B (PIB) is reliably higher in patients with mild AD than in healthy controls [22-24] and appears to reach a plateau in the early stages of AD with no further significant progression over time [20, 21, 25, 26]. CSF biomarkers such as Aβ and tau reflect the brain pathology in patients with AD [27]. Several studies have shown markedly higher levels of CSF T-tau and P-tau in these patients, accompanied by markedly lower levels of Aβ 1-42 (Aβ42), than in healthy controls (for reviews see papers by Blennow and Hampel) [28-30]. However, longitudinal studies have shown stable CSF levels of T-tau, P-tau and Aβ42 in AD [31, 32], suggesting that it should be possible to detect even minor changes induced by treatment using these markers.

We showed that CSF Aβ 1-40 (Aβ40) levels increased in AD patients in whom brain PIB retention decreased in response to 3-6 months of (–)-phenserine therapy [33]. We also observed an increase in rCMRglc and improvements in cognitive function [33]. Positive effects of phenserine on cognitive function have been reported in AD patients [34].

The aim of this study was to investigate the long-term effects of (–)-phenserine (phenserine) on brain glucose metabolism and changes in CSF Aß and tau biomarkers in patients with AD treated with phenserine for up to 12 months..

Methods

Study design

This was a 12-month study involving 20 subjects with mild AD. The initial first 3-month period was a double blind, placebo-controlled, randomized period. The subjects were grouped according to the treatment they received; placebo group (*n*=10) and phenserine group (*n*=10). The second 3-month period (3 to 6 months) was an open-label extension phase, during which subjects in the placebo group received 5 mg donepezil daily and subjects in the phenserine group remained on phenserine (15 mg b.i.d).[This part of the study has been reported in ref 33].

The present study focus on effects of phenserine after 12 months treatment where all subjects had received phenserine treatment (15 mg b.i.d) for totally 6 months (in addition to earlier placebo/donepezil treatment) and 12 months respectively.

Patients

Twenty subjects with a diagnosis of mild AD (mini-mental state examination [MMSE] score ≥ 21) were recruited from the Department of Geriatric Medicine, Karolinska University Hospital Huddinge, Stockholm, Sweden. All subjects had been referred for assessment of dementia because of a memory problem and had undergone a thorough clinical investigation including medical history, cognitive screening (MMSE), physical and neurological examination, laboratory blood tests, apolipoprotein E (*APOE*) genotyping, neuropsychological assessment, lumbar puncture and magnetic resonance imaging/computed

tomography scans. The diagnosis of AD was made by exclusion of other dementia diseases, in accordance with criteria from the National Institute of Neurological and Communication Disorders and Stroke – Alzheimer's disease and Related Disorders Association (NINCDS-ADRDA) [35].

All patients and their responsible caregivers provided written informed consent before participation in the study, which was conducted according to the declaration of Helsinki and subsequent revisions and was approved by the Ethics Committee of Karolinska University Hospital Huddinge and the Faculty of Medicine and Radiation Hazard Ethics Committee of Uppsala University Hospital, Sweden.

Assessment of Safety

Safety was assessed throughout the study. The safety variables monitored included adverse events (AEs), vital signs, physical examination, laboratory safety screen, and electrocardiogram.

PET methods

PET studies were performed at the PET Center, Uppsala Imanet AB, Imanet GE Healthcare. ^{18}F-FDG PET studies were performed at baseline and after 12 months. FDG data after 3 and 6 months have already been published [33]. The patients also underwent Aβ imaging with ^{11}C-PIB at baseline and after 3 and 6 months; see Kadir et al. for the methodology [33].

PET scanning

The PET scans were performed using Siemens ECAT EXACT HR+ scanners (CTI PET-systems Inc.), with an axial field of view of 155 mm, providing 63 contiguous 2.46 mm slices with 5.6 mm transaxial and 5.4 mm axial resolution. The patients were scanned after fasting for 4 hr under resting conditions in a dimmed room. The orbitomeatal line was used to center their heads for the scan. The data were acquired in three-dimensional mode. The scanner protocol for transmissions and reconstructions has been described previously [36]. The

emission scans consisted of 7 frames with a progressive increase in frame duration (1×60 s, 1×1140 s and 5×300 s) and a total duration of 45 minutes. The average tracer dose administered was 210 ± 64.8 Mbq (mean ± SD).

Regions of interest

A set of standardized regions of interest (ROI) were defined using the Scanditronix program (IDA, Images Display and Analyses GE 1994). All ROI were paired for the right and left hemispheres, except for the pons and whole brain. The ROI placement procedure has been described in detail earlier [37].

In this paper we have focused on the analysis of the ROI that were considered to be most critical for AD. The PET data were grouped by ROI into: (i) the frontal cortex (including the frontal, anterior cingulate and frontal association cortices), (ii) the parietal cortex, (iii) the parietotemporal cortex and (iv) the temporal cortex (including the posterior/anterior lateral, inferior, and medial temporal cortices).

Data management

Venous arterialized blood samples were obtained for FDG, and parametric maps were generated by means of the Patlak method, using the time course of the tracer in the arterialized venous plasma as an input function [38]. The rCMRglc values were normalized to the pons value (ROI/ref) to allow inter- and intra-individual comparisons [39].

Statistical Parametric Mapping methods

Voxel-based analysis with statistical parametric mapping (SPM5) (Wellcome Department of Cognitive Neurology, Institute of Neurology, London, UK), which was implemented using Matlab 7.1 (MathWorks Inc., Sherborn, MA), was used to compare the rCMRglc at baseline and after 6-12 months' phenserine treatment. The individual Patlak images were normalized using each patient's individual pons ROI value. All PET images were spatially normalized into the Montreal Neurological Institute standard template (McGill University, Montreal,

Canada) to remove inter-scan and inter-subject anatomical variability. Spatially normalized images were smoothed by convolution, using an isotropic Gaussian kernel with 8 mm full-width at half-maximum. The voxel-wise paired t-test was used to compare baseline with follow-up measurements. Differences were considered significant at $p < 0.05$ (corrected for multiple comparisons using the family wise error rate) and an extent threshold of 100 voxels.

CSF biomarkers

CSF samples (12 mL) were collected by lumbar puncture at baseline and after 12 months of treatment. All samples were taken in the morning or 2-4 hours after intake of morning medications. CSF samples were collected from 14 of the 20 patients after 12 months of treatment.

The CSF samples were collected in polypropylene tubes, and gently mixed to avoid gradient effects. Those with more than 500 erythrocytes/µL were excluded. All samples were centrifuged, aliquoted and stored in polypropylene tubes at -80 ºC pending biochemical analyses, without being thawed and re-frozen.

Levels of Aβ42, total tau (T-tau) and phosphorylated tau (P-tau181) in CSF were analyzed using the INNO-BIA AlzBio3 assay, as described previously in detail [40].

Levels of CSF α- and β-secretase-cleaved soluble APP (α-sAPP and β-sAPP, respectively) were measured using Meso Scale Discovery (MSD®) electrochemiluminescence detection technology (sAPPα/sAPPβ multiplex assay; Meso Scale Discovery, Gaithersburg, Maryland) and a SECTOR™ Imager 2400 instrument, following the instructions of the manufacturer. The β-sAPP assay is based on a β-sAPP-specific capture antibody, with recombinant β-sAPP as standard, and an N-terminal APP-specific SULFO-TAG™-labeled antibody as detector. The α-sAPP assay is based on 6E10 monoclonal antibody (MAb) as capture antibody, with recombinant α-sAPP as standard, and an N-terminal APP-specific SULFO-TAG™-labeled antibody as detector.

Levels of CSF Aβ 1-38 (Aβ38), Aβ40 and Aβ42 were measured using the MSD Human/Rodent (4G8) Abeta Triplex Assay as described by the manufacturer (Meso Scale Discovery, Gaithersburg, MD, USA). This assay employs C-terminal-specific antibodies to capture Aβx-38, Aβx-40 and Aβx-42, and the Aβ mid-domain 4G8 antibody in combination with a SULFO-TAG-labeled anti-4G8 to quantify them.

Cognitive function

Neuropsychological tests of episodic memory [Word Recall (2 and 5 sec presentation rate), Word Recognition-d (2 and 5 sec presentation rate)], attention [Digit Symbol, TMT-A (time)] and visuospatial ability [Clock Recognition] were carried out at baseline and after 12 months' treatment. The neuropsychological procedure has been described in detail previously [33]

In order to make comparisons between the neuropsychological test results, all the raw cognitive scores were z-transformed (to z-scores) using reference data from the Geriatric Clinic, Karolinska University Hospital Huddinge, Stockholm, Sweden [41].

Statistical analysis

Data are expressed as mean values and the standard error of the mean (SEM). The equivalence of the demographic variables of the phenserine 6-month and 12-month groups was checked by means of one-way analysis of variance. Wilcoxon's signed rank test was used to compare baseline values versus those at other time points. This non-parametric method was chosen for within-group analysis because of the small sample size and because we could not assume that all parameters were normally distributed. Two-tailed Pearson's correlation coefficients, and Spearman's Rank correlations when appropriate, were used in the correlation analysis, which was then visualized graphically using simple regression plots. All statistical tests were two-tailed at the 0.05 level of significance and no corrections for multiple comparisons were made because this was an explorative type of study.

Results

Demography

The demographic characteristics of the patients are presented in **Table 1**. No significant differences were observed between the 6-month and 12-month phenserine treatment groups.

Changes in CSF biomarkers

A significant increase from baseline in levels of CSF Aβ40, α-sAPP and β-sAPP (all $p<0.05$) was observed after 6-12 months' treatment. This increase was more pronounced in the patients treated with phenserine for 12 months than in those receiving only 6 months treatment (**Table 2**). No significant differences from baseline were observed in CSF Aβ42 levels, P-tau levels or the Aβ42/40 ratio after treatment. A significant increase in CSF T-tau levels was observed after treatment ($p< 0.01$); this was more pronounced in patients who were treated with phenserine for 6 months after having received placebo for 3 months and donepezil for 3 months.

Changes in rCMRglc

The SPM analysis did not show any significant changes in the rCMRglc after 6-12 months of phenserine treatment compared to baseline, indicating that the rCMRglc was stabilized by long-term phenserine treatment.

However, the ROI-based analysis indicated a –2 to –5% reduction in rCMRglc from baseline after 6-12 months of phenserine treatment, reaching significance in the temporal cortex for the total phenserine group (6-12 months' treatment), and in the right frontal cortex in patients receiving 12 months of phenserine (suppl. **Table 1**).

The changes in rCMRglc in all four cortical regions correlated positively with the changes in CSF Aβ40, α-sAPP and β-sAPP concentrations. A significant positive correlation

was observed between changes in rCMRglc in the left frontal cortex and CSF Aβ40 levels (r = 0.52, p<0.03, *one-tailed*, n = 14, **Fig 1A**).

In contrast, the changes in rCMRglc in all four cortical regions correlated negatively with the changes in CSF Aβ42, CSF Aß42/40, T-tau and P-tau. A significant negative correlation was observed between changes in rCMRglc in the parietotemporal cortex and CSF Aβ42 (r = -0.56, p< 0.03, **Fig 1B**), as well as between changes in rCMRglc in the left frontal cortex and CSF Aß42/40 (r = -0.49, p<0.04, one tailed, **Fig 1C**).

These observations indicate that AD patients with increased glucose metabolic rates after phenserine treatment also had increased CSF Aβ40 levels, decreased CSF Aβ42 levels, and decreased Aβ42/40 ratios.

Changes in cognitive function

No statistically significant changes from baseline, except for those in the visuospatial ability test, were observed after 6-12 months of phenserine treatment (Suppl. Table 2).

Correlation between CSF biomarkers and cognitive function

After 6-12 months of phenserine treatment, a significant positive correlation was observed between the increase in CSF levels of Aβ40 and improvements in the attention test scores (r = 0.58, p<0.03, **Fig 2A**).

Significant negative correlations were also observed between changes in CSF Aβ42 levels (r = -0.64, p<0.01, **Fig 2B**), CSF Aβ42/40 ratios (r = -0.75, p<0.001, **Fig 2C**) and episodic memory. This finding suggests that AD patients with decreased CSF Aβ42 levels after phenserine treatment also have improvements in episodic memory.

Correlation between rCMRglc and cognitive function

After 6-12 months of phenserine treatment, the changes in rCMRglc in all four cortical regions correlated positively with the composite z-score for the seven neuropsychological tests. Significant positive correlations were observed in the right parietal (r = 0.51, p<0.02), left parietal (r = 0.48, p<0.04), left parietotemporal (r = 0.51, p<0.02), right temporal (r = 0.46, p<0.04) and left temporal cortices (r = 0.55, p<0.01, **Fig 3**). These findings demonstrate that AD patients with increased cortical rCMRglc after long-term phenserine treatment also showed improved cognitive function in comparison with AD patients with no change or decline in rCMRglc.

Correlation between CSF biomarkers, cognitive function and cortical PIB retention

PIB imaging were in the initially part of the study [33] performed at baseline and after 3 and 6 months of treatment with phenserine to obtain a representation of the fibrillar Aβ load **Fig 4** illustrates the parametric images of cortical PIB retention in two patients with mild AD, at baseline and after 3 or 6 months of phenserine treatment.

When the changes in PIB retention in the left parietal cortex at 3 months were plotted against the CSF biomarker levels after 12 months treatment , a significant negative correlation was observed between PIB retention and CSF Aβ40 levels (r = -0.71, p<0.003, Fig 5A), a significant positive correlation with CSF Aß42/40 (r=0.58, p<0.03, Fig 5B) as well as negative correlation with episodic memory tet (r=-0.41, one tailed, Fig 5C) .

Discussion

Intense multidisciplinary research efforts during recent decades have provided detailed knowledge on the molecular pathogenesis of AD, which has been translated into promising novel candidate drugs with putative disease-modifying effects. Aβ plaque accumulation, tau

phosphorylation, inflammation, synaptic abnormalities and cell death are all thought to contribute to AD and are potential targets for pharmacological manipulation [1, 8, 42]. Many of clinical trials performed during the recent 10 years have however been disappointing with frequent failures [43]. Dynamic changes to AD biomarkers during the time course of the disease have been discussed and demonstrated in several studies [22, 44-46]. The increase in Aβ plaque accumulation in the brain and decrease in Aβ42 levels in the CSF appear to occur early in the preclinical AD stages, while a decline in brain glucose metabolism reflecting changes in brain functional activity is more closely related to cognitive impairment and disease severity [21, 22, 47-49]. In this study, a multi-biomarker approach (PET imaging, CSF biomarkers and cognitive function tests) was applied to investigate the inter-relationships between brain glucose metabolism, and levels of Aβ plaque in the brain and the CSF, CSF tau and p-tau in relation to cognitive function in patients with mild AD to evaluate the effects of 12 months of treatment with phenserine.

The main aim of disease-modifying drugs is to inhibit brain Aβ production and aggregation and to increase Aβ clearance from the brain. The (–)-phenserine enantiomer, a derivative of physostigmine, reduces APP and Aβ concentrations by decreasing the translation of APP mRNA [50]. In addition to providing symptomatic relief in patients with AD by augmenting cholinergic function, phenserine could also modify the disease activity by decreasing the generation of APP and Aβ, a mechanism implicated by the Aβ hypothesis in AD as a potential pathological process leading to disease progression.

In this study, significant increased levels of CSF Aβ40, α-sAPP and β-sAPP were demonstrated after –up to 12 months of phenserine treatment. Previous follow-up studies in AD patients have shown either unaltered levels [51, 52] or mildly elevated levels [53] of α-sAPP and β-sAPP in sporadic AD, and have also indicated that α–sAPP and β–sAPP levels are tightly correlated with each other [52-54]. Recently, it was shown that CSF Aβ40,

α−sAPP and β−sAPP levels are decreased in severe AD, which could relate to neuronal loss or reduced brain metabolism in the end stages of the disease [55]. In our study, we observed that the elevated CSF Aβ40 and sAPP levels correlated positively with both increased rCMRglc and improved cognitive function but not with Aβ42 levels, strengthening the hypothesis that the release of these molecules to the extracellular fluid may be influenced by brain metabolism and/or neuronal function.

Experimental studies in primary rat neurons treated with rivastigmine under media conditions showed dose-dependent increase in the levels of synaptic markers such as synaptophysine and SNAP-25 suggesting and neuroprotective effect [56]. Furthermore, rivastigmine increased sAPPα and lowers Aß levels in primary cell cultures as well as sAPP in rat CSF following 21 days treatment with rivastigmine suggesting neuronal survival [57].

A recent study of the effects of 10 days' treatment with posiphen, the (+)-enantiomer of phenserine, in patients with mild cognitive impairment (MCI) showed lowered CSF levels of α−sAPP and β−sAPP [~~56~~58], which could be explained by acute inhibition of APP translation. Our finding of elevated sAPP levels during long-term treatment could suggest that (–)-phenserine changes the release of these APP fragments to the CSF, and it is tempting to speculate that the increase relates to improved brain metabolism. On the other hand, modification of the degradation or clearance of these sAPP fragments in AD patients could also account for our results. An important observation in this study was that the elevation of CSF Aβ40 and sAPP levels was more pronounced in the group treated with phenserine for 12 months than in the group treated for initially treated with placebo and donepezil followed by 6 months phenserine treatment. This observation could indicate that longer phenserine treatment is needed to see a change in the CSF biomarkers.

Experimental studies have shown that overexpression of Aβ40 in transgenic mice does not result in brain Aβ deposition, whereas expression of Aβ42 is known to be essential

for Aβ deposition [57, 58]59,60]. In addition, both *in vitro* and *in vivo* studies have indicated that Aβ40 could have an inhibitory effect on Aβ deposition and could also possibly have neuroprotective effects [59, 60][61-62]. Increased levels of Aβ40 in the brains of Tg2576 or BRI-Aβ42A mice appear to protect against Aβ pathology [6163]. Results from transgenic mice expressing wild-type and various mutant forms of APP also suggest that increased Aβ40 levels could reduce Aβ deposition [5860]. It has also been shown that high Aβ40 levels significantly delay the age of onset of clinical manifestations of familial AD (FAD)-linked presenilin1 (PSEN1) mutations [6264]. These lines of evidence suggest that converting Aβ42 to Aβ40 may be a potential strategy for development of an AD therapy. Finally, recent data in mice suggest that Aβ40 could have anti-inflammatory properties [6365].

In this study we found that subjects with decreased Aβ42 levels after phenserine treatment showed increased rCMRglc. It is still not entirely clear whether alterations to CSF protein levels can reflect neuronal or synaptic dysfunction in AD, since few studies are available. The significant positive relationship between CSF Aβ42 levels and brain glucose metabolism in AD patients found by Vukovich et al. [6466] suggests that low CSF Aβ42 levels are associated with decreased rCMRglc. Similar positive correlations between CSF Aβ42 and rCMRglc have also been observed in two other studies in AD patients [6567] and in healthy controls [6668]. The negative correlation between CSF Aβ42 and rCMRglc in phenserine-treated patients in this study suggests a possible anti-Aβ effect of phenserine treatment.

CSF Aβ40 is thought to be the most abundant Aβ isoform in the CSF [6769]. While no major change has been detected in CSF Aβ40 levels in patients with AD or MCI, a marked decrease (more pronounced than the reduction in CSF Aβ42) has been observed in the Aβ42/Aβ40 ratio [68, 69]70,71]. We observed a slight decrease in the CSF Aβ42/Aβ40 ratio following phenserine treatment. Patients with low CSF Aβ42/40 ratios showed increased

rCMRglc and improved cognitive function after long-term treatment as well as decreased CSF Aβ42 levels.

Clinical trials of anti-Aβ compounds in AD patients have shown various effects on CSF biomarkers. The γ-secretase inhibitor semagacestat (LY450139) increased levels of the shorter Aβ isoforms (Aβ14, Aβ15 and Aβ16) in the CSF of AD patients, but did not show any effect on Aβ42 or Aβ40 levels [~~70~~72]. A decrease in CSF Aβ42 levels was seen in AD patients after 12 weeks of treatment with PBT2, a metal-protein attenuating compound (MPAC) [~~71~~73]. Increases to the total levels of CSF Aβ were reported following treatment of AD patients over a 12-week period with the monoclonal antibody against Aβ (solanezumab, LY2062430) [~~72~~74]. Treatment with the anti-ß monoclonal antibody bapineuzumab was recently reported to decrease tau and ptau levels with no effects on CSF Aβ40 or Aβ42 [~~73~~75]. The decrease in CSF tau was observed in APOE e 4 carriers [12].

In this study, we observed a significant increase in CSF T-tau levels following phenserine treatment, while no significant changes were seen in CSF p-tau levels. The changes in CSF T-tau levels correlated negatively with both rCMRglc and attention test scores while corresponding p--tau levels were positively correlated with CSF Aβ42 levels and negatively correlated with cognitive function following 12 months of phenserine treatment (data not shown). Increased CSF levels of T-tau could indicate more intense neurodegeneration, but recent data also suggest that tau secretion from cells into the CSF could also be stimulated by Aβ in the absence of neuronal death [~~74~~76]. Further, *in vitro* treatment of SH-SY5Y cells with ChEIs and nicotinic agonists caused an increase in tau levels [~~75~~77]. The exact molecular mechanism by which ChEI induces changes in tau remains unclear. Rivastigmine treatment of AD patients showed no significant changes in CSF T-tau levels after one year of follow-up [~~76~~78], while a significant increase in T-tau levels was observed in tacrine-treated AD patients, which was more pronounced in *APOE* ε4

carriers [~~76~~78]. The presence of *APOE* ε4 increases progression of AD and increases CSF tau levels [~~77~~79]. Clearly, the increased CSF T-tau levels observed during phenserine treatment in our study must be examined closely in future investigations.

Measurement of rCMRglc with 18F-FDG reflects the functional activity of the brain. A noticeable decline in rCMRglc occurs over the course of a year in AD patients [~~78-80~~80-82-].. A significantly greater bilateral decline in rCMRglc was recently observed in the posterior cingulate, parietal, temporal, frontal and occipital cortices of AD patients than in those of healthy controls after 12 months of follow-up [~~81~~83]. In agreement with earlier studies in other AChEIs including donepezil [80], rivastigmine [79], tacrine [~~82~~84] and galantamine [~~83~~85] we observed with pheserine a long-term stabilization of rCMRglc. We also found in AD patients after phenserine treatment a positive correlation between rCMRglc and cognitive function.

Aβ imaging is expected to be useful in the evaluation of disease-modifying anti-Aβ therapeutic agents in patients with AD. Studies of monoclonal anti-Aβ antibody treatment have demonstrated decreases in ^{11}C-PIB after 78 weeks of treatment with bapineuzumab (AAB-001) [~~84~~86] or gantenerumab [~~85~~87], while no effects on CSF Aβ levels [~~86~~88] or cognition [~~87~~89,12] have been observed. It is possible that the current failure to find clinical effects of anti-Aβ disease-modifying drugs is because the drugs have been tested in patients with very advanced disease, or because the treatment duration may have been too short, especially for disease modifying effects [43]. Despite this, brain imaging and CSF biomarkers could be used to indicate that the tested drug affects pathological disturbances. It could therefore be of value to further evaluate biomarkers in drug trials, and in the very early stages of AD. The use of Aβ imaging and measurement of CSF biomarker levels when including patients in clinical trials, and when testing preventative drugs in cognitively normal subjects with AD pathology is worth consideration ~~[88, 89]~~90,91].

An important limitation of this study is the relatively large number of statistical analyses performed on the PET parameters in relation to the CSF biomarkers and cognitive scores [~~90~~92]. These analyses were not corrected for multiple comparisons, which may mean that some of the results could have been obtained by chance. However, result patterns were taken into consideration rather than isolated findings. Nevertheless, these features necessitate the use of caution when interpreting the data.

In conclusion, long-term phenserine (30 mg/day) treatment significantly increased CSF Aβ40 and sAPP α and β levels from baseline measurements. The increase in CSF Aβ40 and sAPP levels correlated positively with increased rCMRglc and cognitive function. This study of the effects of phenserine illustrates interactive effect mechanisms between CSF biomarkers, functional imaging and cognition and highlights the potential roles for PET and CSF biomarkers in the assessment of the efficacy of AD therapeutics.

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Figure 1

Positive correlation between percentage changes in rCMRglc in the left frontal cortex and changes in CSF Aβ40 levels at 12 months (A). Negative correlations between percentage changes in rCMRglc in the left parietotemporal cortex and changes in CSF Aβ42 levels at 12 months (B); changes in rCMRglc in the left frontal cortex and changes in CSF Aβ42/40 ratio at 12 months (C). All values on both axes are changes from individual baseline values. Filled squares = phenserine 12 months treated group; open circles = phenserine 6 months treated group.

Figure 2

Positive correlations between changes in CSF Aβ40 levels and changes in composite attention z-score (Digit Symbol and Trail Making Test-A) at 12 months (A). Negative correlations between changes in CSF Aβ42 levels and changes in composite episodic memory z-score (Word recall and Word recognition-d) at 12 months (B); changes in CSF Aβ42/40 ratio and changes in composite episodic memory z-score at 12 months (C). At 12 months, all values are absolute value changes from individual baseline values. Filled squares = phenserine 12 months treated group; open circles = phenserine 6 months treated group.

Figure 3

Positive correlation between changes in rCMRglc in the left temporal cortex and the composite z-score in seven neuropsychological sub-tests at 12 months. On the x axis, rCMRglc, percentage changes from individual baseline values at 12 months, and on the y axis, absolute values of composite neuropsychological z-scores at 12 months. Filled

squares = phenserine 12 months treated group; open circles = phenserine 6 months treated group.

Figure 4

Negative correlation between changes in PIB retention in the left parietal cortex at 3 months and changes in CSF Aβ40 concentrations at 12 months (A). Positive correlation between changes in PIB retention in the left parietal cortex at 3 months and changes in CSF Aβ42/40 ratio at 12 months (B). Negative correlation between changes in PIB retention in the left parietal cortex at 3 months and changes in CSF composite episodic memory z-score (Word recall and Word recognition-d) at12 months (C). All values in both axis are changes from individual baseline values. Filled squares for PIB data are 3 months phenserine treatment and for CSF and cognition are 12 months phenserine treated patients. Open circles for PIB data placebo treated patients and for CSF and cognition 6 months phenserine treated patients.

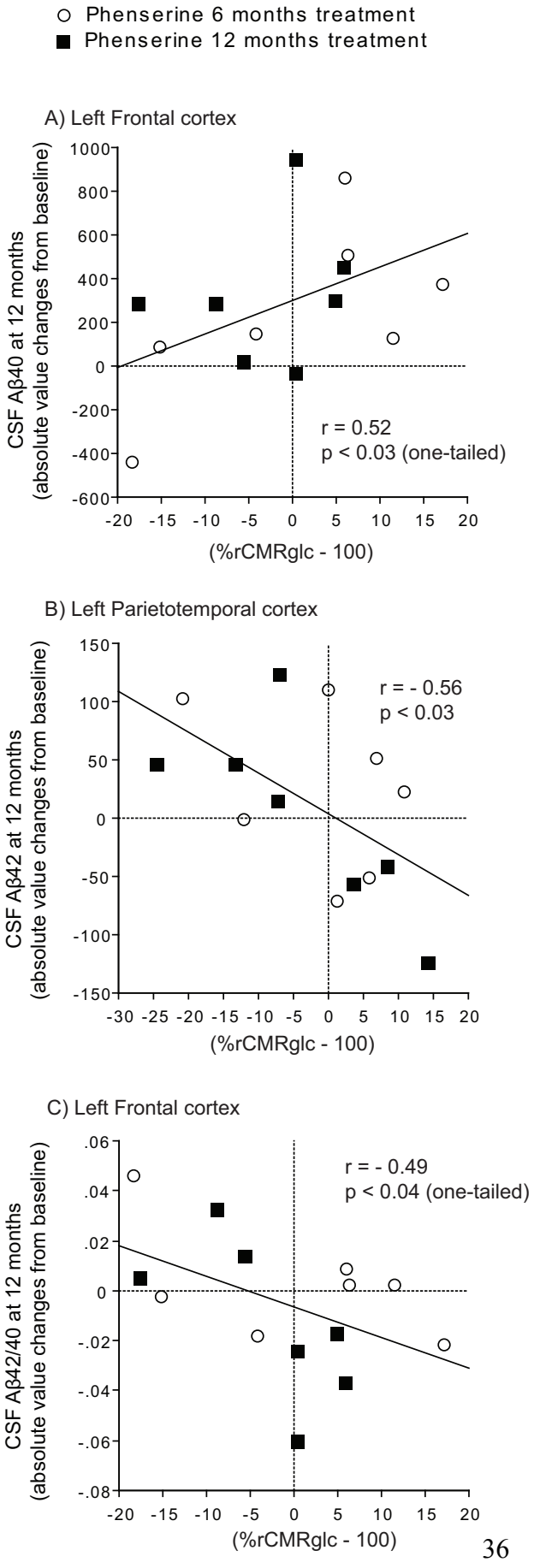
Phenserine 6 months treatment
Phenserine 12 months treatment
A) Left Frontal cortex
CSF Aβ40 at 12 months
(absolute value changes from baseline)
1000
800
600
400
200
0
-200
-400
-600
-20 -15 -10 -5 0 5 10 15 20
(%rCMRglc - 100)
r = 0.52
p < 0.03 (one-tailed)
B) Left Parietotemporal cortex
CSF Aβ42 at 12 months
(absolute value changes from baseline)
150
100
50
0
-50
-100
-150
-30 -25 -20 -15 -10 -5 0 5 10 15 20
(%rCMRglc - 100)
r = - 0.56
p < 0.03
C) Left Frontal cortex
CSF Aβ42/40 at 12 months
(absolute value changes from baseline)
.06
.04
.02
0
-.02
-.04
-.06
-.08
-20 -15 -10 -5 0 5 10 15 20
(%rCMRglc - 100)
r = - 0.49
p < 0.04 (one-tailed)

Figure 1

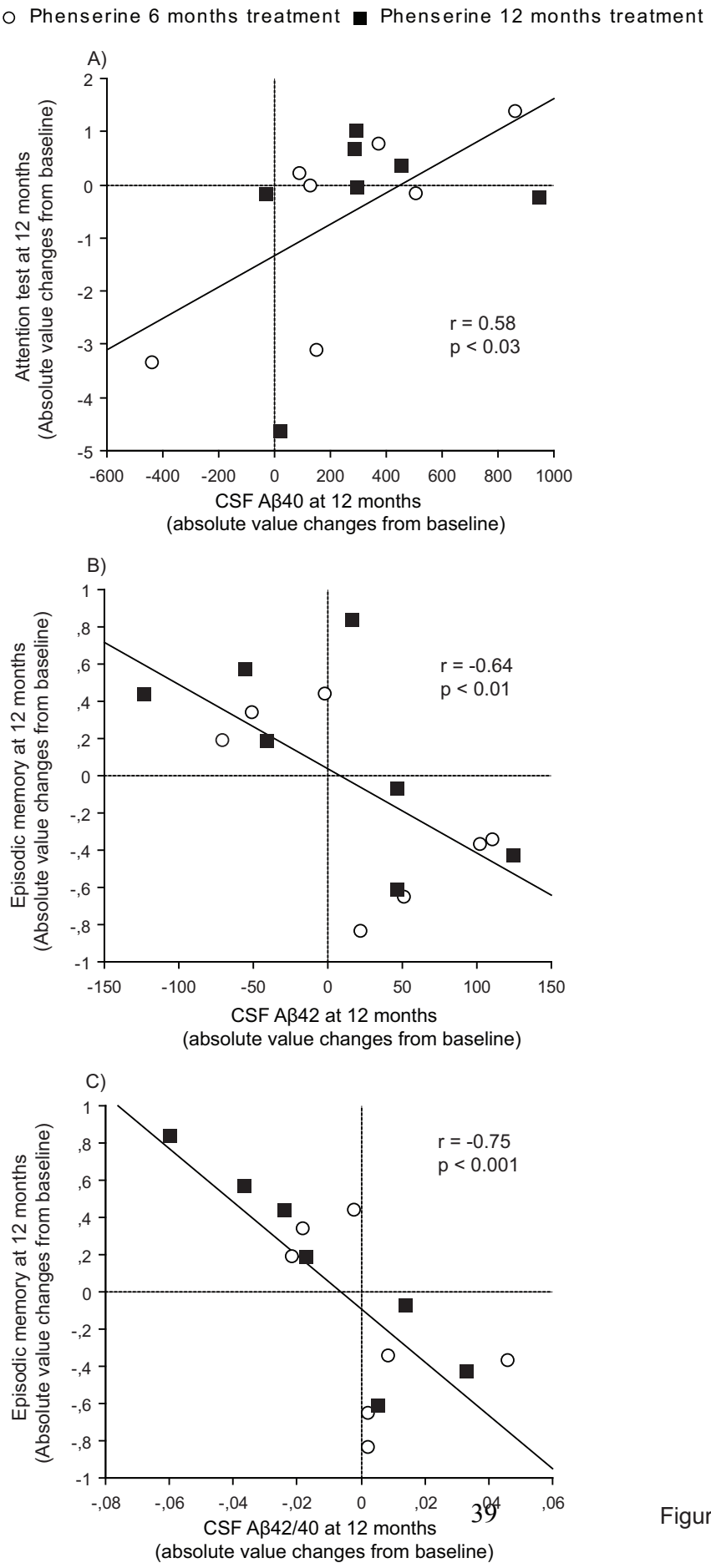
○ Phenserine 6 months treatment ■ Phenserine 12 months treatment
A)
Attention test at 12 months
(Absolute value changes from baseline)
r = 0.58
p < 0.03
CSF Aβ40 at 12 months
(absolute value changes from baseline)
B)
Episodic memory at 12 months
(Absolute value changes from baseline)
r = -0.64
p < 0.01
CSF Aβ42 at 12 months
(absolute value changes from baseline)
C)
Episodic memory at 12 months
(Absolute value changes from baseline)
r = -0.75
p < 0.001
CSF Aβ42/40 at 12 months
(absolute value changes from baseline)

Figure 2

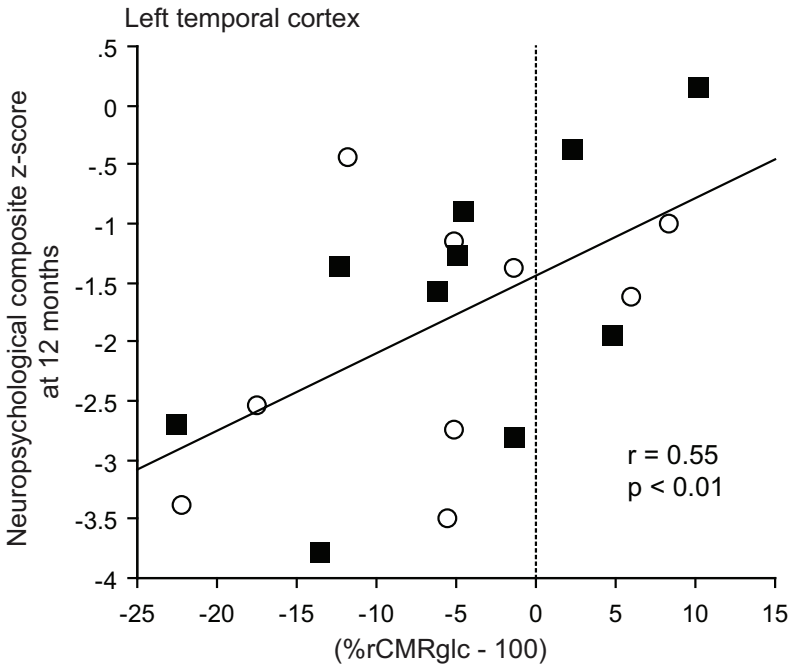
Left temporal cortex
Neuropsychological composite z-score at 12 months
.5
0
-.5
-1
-1.5
-2
-2.5
-3
-3.5
-4
-25
-20
-15
-10
-5
0
5
10
15
(%rCMRglc - 100)
r = 0.55
p < 0.01

Figure 3

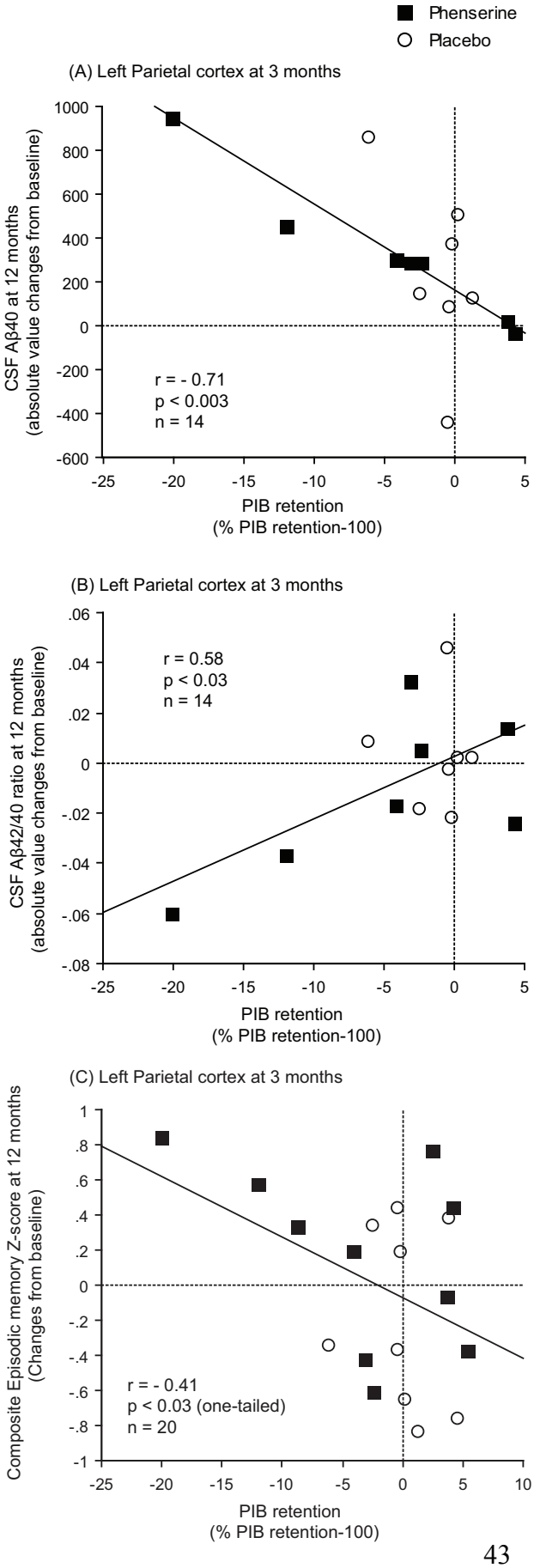

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Table 1:

Demographic data for patients with mild Alzheimer's disease entering the 12-month study. Data are presented as means ± SE

	All subjects	Phenserine 6-month treatment group*
Total subjects (*n*)	20	10
Male/Female	5/15	3/7
Age (years)	67.9 ± 1.9	69.8 ± 2.7
Education (years)	11.8 ± 0.9	11.5 ± 1.2
Duration of disease (years)	3.7 ± 0.4	3.7 ± 0.6
First degree relative with AD (yes/no)	6/14	2/8
APOE ε4 carriers (++/-+/--)	6/12/1	3/7/0
MMSE score at baseline	24.0 ± 0.6	23.0 ± 0.8
Composite cognitive z-score**	-1.7 ± 0.3	-2.0 ± 0.5
Attention z-score	-2.4 ± 0.6	-3.1 ± 1.1
Episodic memory z-score	-1.7 ± 0.1	-1.8 ± 0.2
Visuospatial ability z-score	-1.1 ± 0.4	-1.0 ± 0.5

APOE = apolipoprotein E; MMSE = Mini-Mental State Examination
*This patients group received placebo for the first 3 months followed by donepezil for 3 months and phenserine for 6 months. ** The composite cognitive z-score reflects the amalgamated attention, episodic memory and visuospatial ability test scores.

Table 2: Absolute values of CSF biomarkers in patients with mild Alzheimer's disease at baseline and after treatment with oral phenserine. The first 6-months' data, for patients receiving phenserine or placebo and then donepezil, are taken [33].Data are expressed as means ± SE; minimum and maximum values are given in parentheses.

	Phenserine (12 months) (n = 7)			Placebo (3months), Donepezil (3 months), Phenserine (6 months) (n = 7)			Total Phenserine treatment group (6 or 12 months) (n = 14)	
Biomarkers	Baseline	6 months	12 months	Baseline	6 months	12 months	Baseline	6 or 12 months
CSF Aβ38 (pg/ml)	904 ± 133 (414-1419)	942 ± 131 (414-1383)	969 ± 155 (522-1592)	1245 ± 228 (481-1918)	1241 ± 205 (631-1843)	1152 ± 207 (389-1756)	1075 ± 135 (414-1918)	1061 ± 127 (389-1756)
CSF Aβ40 (pg/ml)	***3532 ± 364*** (2164 - 5041)	3525 ± 236 (2858 - 4596)	***3853 ± 283**** (3109 - 5005)	3961 ± 324 (2474-4913)	3845 ± 281 (3060-4965)	4199 ± 339 (2916-5284)	***3747 ± 241*** (2164-5041)	***4027 ± 218**** (2916-5284)
CSF Aβ42 (pg/ml)	374 ± 54 (105- 522)	381 ± 41 (198-512)	375 ± 30 (229-477)	383 ± 44 (233-564)	407 ± 30 (329-543)	405 ± 20 (342-493)	378 ± 34 (105-564)	391 ± 18 (229-493)
CSF Aβ42/40	0.112 ± 0.021 (0.033-0.213)	0.110 ± 0.014 (0.060-0.154)	0.100 ± 0.011 (0.066-0.154)	0.096 ± 0.007 (0.072-0.122)	0.107 ± 0.007 (0.080-0.129)	0.098 ± 0.004 (0.082-0.118)	0.104 ± 0.011 (0.033-0.213)	0.099 ± 0.006 (0.066-0.154)
CSF Aβ42/38	0.497 ± 0.120 (0.084–1.116)	0.488 ± 0.120 (0.143-1.161	0.453 ± 0.089 (0.250-0.915)	0.353 ± 0.047 (0.193-0.527)	0.375 ± 0.064 (0.198-0.600)	0.437 ± 0.086 (0.243-0.882)	0.425 ± 0.065 (0.084-1.116)	0.445 ± 0.059 (0.243-0.915)
CSF Aβ40/38	4.15 ± 0.34 2.57-5.23	4.13 ± 0.50 (2.39-6.48)	4.33 ± 0.39 (3.14-5.96)	***3.73 ± 0.52*** (2.41-5.86)	3.56 ± 0.48 (2.43-4.97)	***4.29 ± 0.64**** (2.95-7.50)	3.90 ± 0.31 (2.41-5.86)	4.30 ± 0.36 (2.95-7.50)
T-tau (pg/ml)	567 ± 137 (172-1194)	565 ± 129 (224-1131)	710 ± 146 (266-1316)	***584 ± 115*** (166-1110)	616 ± 106 (309-1057)	***656 ± 113**** (240-1120)	***576 ± 86*** (166-1194)	***683 ± 89**** (240-1315)
P-tau (pg/ml)	94 ± 11 (68-146)	101 ± 10 (65-138)	96 ± 9 (62-129)	90 ± 9 (58-124)	89 ± 5 (69-113)	95 ± 8 (62-122)	92 ± 7 (58-146)	96 ± 6 (63-129)
CSF α-sAPP (ng/ml)	***479 ± 40*** (317-589)	500 ± 54 (301-740)	***586 ± 66**** (352-805)	655 ± 75 (347-890)	699 ± 83 (379-981)	714 ± 93 (413-1093)	***567 ± 47*** (317-890)	***650 ± 58**** (352-1093)
CSF β-sAPP (ng/ml)	***425 ± 28*** (292-509)	436 ± 37 (287-607)	***482 ± 47**** (311-630)	521 ± 46 (308-682)	532 ± 40 (334-648)	544 ± 47 (376-692	***473 ± 29*** (292-682)	***513 ± 33**** (311-692)

*p<0.05, **p<0.01 versus baseline.

Suppl. Table 1: Regional cerebral glucose metabolism (rCMRglc, normalized to the pons) in cortical brain regions of patients with mild Alzheimer's disease at baseline and after 6 or 12 months of treatment with oral phenserine titrated to 15 mg twice daily. Data are expressed as means ± SE. Post-treatment rCMRglc values were calculated as percentage changes from the baseline (pretreatment) value using the following formula:

	Total group		Phenserine 6 months' treatment		Phenserine 12 months' treatment	
Brain regions	Baseline (n = 19)[a]	6-12 months (n = 19)	Baseline (n = 9)	6 months (n = 9)	Baseline (n = 10)	12 months (n = 10)
Frontal cortex rt	1.43 ± 0.04	1.37 ± 0.04 (-4%)	1. 40 ± 0.07	1.37 ± 0.07 (-2%)	***1.46 ± 0.05***	***1.37 ± 0.04 (-6%)***
Frontal cortex lt	1.42 ± 0.04	1.39 ± 0.04 (-2%)	1.41 ± 0.06	1.40 ± 0.06 (-0.3%)	1.42 ± 0.04	1.38 ± 0.05 (-3%)
Parietal cortex rt	1.27 ± 0.05	1.22 ± 0.06 (-4%)	1.28 ± 0.09	1.25 ± 0.11 (-3%)	1.26 ± 0.06	1.19 ± 0.06 (-5%)
Parietal cortex lt	1.22 ± 0.05	1.18 ± 0.06 (-4%)	1.23 ± 0.08	1.21 ± 0.10 (-2%)	1.22 ± 0.07	1.15 ± 0.06 (-5%)
Parietotemporal cortex rt	1.22 ± 0.05	1.18 ± 0.06 (-3%)	1.24 ± 0.08	1.21 ± 0.30 (-3%)	1.21 ± 0.07	1.15 ± 0.06 (-3%)
Parietotemporal cortex lt	1.22 ± 0.0	1.16 ± 0.06 (-4%)	1.26 ± 0.08	1.21 ± 0.08 (-4%)	1.18 ± 0.08	1.13 ± 0.09 (-4%)
Temporal cortex rt	***1.09 ± 0.03***	***1.02 ± 0.02 (-5%)****	1.08 ± 0.06	1.02 ± 0.05 (-5%)	1.09 ± 0.04	1.02 ± 0.03 (-5%)
Temporal cortex lt	***1.07 ± 0.03***	***1.00 ± 0.03 (-5%)****	1.07 ± 0.07	1.00 ± 0.05 (-6%)	1.06 ± 0.02	1.01 ± 0.04 (-5%)

%rCMRglc – 100 = ($rCMRglc_{(f)}$ / $rCMRglc_{(b)}$ × 100) - 100, where *f* and *b* indicate the rCMRglc values at follow-up and baseline, respectively.

[a] One of the patients was unable to have the last FDG PET scan; *p<0.05 significant decrease compared with baseline; rt = right; lt = left.

Suppl. Table 2: Cognitive test z-scores for patients with mild Alzheimer's disease at baseline and after 6 or 12 months of oral phenserine treatment. Data are expressed as means ± SE. Raw cognitive scores were z-transformed using reference data from the Geriatric Clinic, Karolinska University Hospital, Huddinge, Stockholm, Sweden. Data in parentheses show the changes to the z-score from baseline at follow-up.

	Total group (n = 20)		Phenserine 6 months' treatment (n = 10)		Phenserine 12 months' treatment (n = 10)	
Biomarkers	Baseline	6-12 months treatment	Baseline	6 months	Baseline	12 months
Composite z-score	-1.72 ± 0.31	-2.06 ± 0.40 (-0.34)	-1.96 ± 0.54	-2.35 ± 0.66 (-0.40)	-1.49 ± 0.30	-1.77 ± 0.48 (-0.27)
Episodic memory z-score	-1.66 ± 0.15	-1.69 ± 0.19 (-0.01)	-1.77 ± 0.20	-1.97 ± 0.25 (-0.20)	-1.58 ± 0.23	-1.42 ± 0.28 (+0.17)
Attention z-score	-2.40 ± 0.59	-2.79 ± 0.74 (-0.40)	-3.10 ± 1.11	-3.60 ± 1.29 (-0.50)	-1.70 ± 0.35	-2.00 ± 0.73(-0.29)
Visuospatial ability z-score	-1.11 ± 0.36	-1.69 ± 0.49 (-0.60)	-1.00 ± 0.51	-1.49 ± 0.70 (-0.50)	-1.19 ± 0.52	-1.89 ± 0.70 (-0.70)*