Plasma Aβ$_{42}$ as Biomarker of Prodromal Alzheimer's Disease Progression in Patients with Amnestic Mild Cognitive Impairment: Evidence from the PharmaCog/E-ADNI Study

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Abstract. It is an open issue whether blood biomarkers serve to diagnose Alzheimer's disease (AD) or monitor its progression over time from prodromal stages. Here, we addressed this question starting from data of the European FP7 IMI-PharmaCog/E-ADNI longitudinal study in amnesic mild cognitive impairment (aMCI) patients including biological, clinical, neuropsychological (e.g., ADAS-Cog13), neuroimaging, and electroencephalographic measures. PharmaCog/E-ADNI patients were classified as "positive" (i.e., "prodromal AD"; $n = 76$) or "negative" ($n = 52$) based on a diagnostic cut-off of Aβ$_{42}$/P-tau in cerebrospinal fluid as well as APOE ε4 genotype. Blood was sampled at baseline and at two follow-ups (12 and 18 months), when plasma amyloid peptide 42 and 40 (Aβ$_{42}$, Aβ$_{40}$) and apolipoprotein J (clusterin, CLU) were assessed. Linear Mixed Models found no significant differences in plasma molecules between the "positive" (i.e., prodromal AD) and "negative" groups at baseline. In contrast, plasma Aβ$_{42}$ showed a greater reduction over time in the prodromal AD than the "negative" aMCI group ($p = 0.048$), while CLU and Aβ$_{40}$ increased, but similarly in the two groups. Furthermore, plasma Aβ$_{42}$ correlated with the ADAS-Cog13 score both in aMCI patients as a whole and the prodromal AD group alone. Finally, CLU correlated with the ADAS-Cog13 only in the whole aMCI group, and no association with ADAS-Cog13 was found for Aβ$_{40}$. In conclusion, plasma Aβ$_{42}$ showed disease progression-related features in aMCI patients with prodromal AD.

Keywords: Amnesic mild cognitive impairment, amyloid-beta peptide, biomarkers, clinical trial, clusterin, PharmaCog project, prodromal Alzheimer's disease

INTRODUCTION

A current hot-spot of clinical research in Alzheimer's disease (AD) deals with the discovery of sensitive, specific, non-invasive, and cost-effective biomarkers useful for the diagnosis or the quantification of illness progression from prodromal stage (amnesic mild cognitive impairment, aMCI) to dementia stage, featuring severe cognitive deficits and disability in self-care and autonomy [1]. According to the current guidelines, as reported in Dubois et al. [1], diagnostic biomarkers of AD include low concentration of Aβ$_{42}$ and high concentration of total tau (T-tau) or phospho-tau (P-tau) in cerebrospinal fluid (CSF), or evidence of significant amyloid deposition and tau aggregation in the brain in maps of positron emission tomography (PET). On the other hand, topographic or progression biomarkers of AD measure atrophy of hippocampus or cerebral cortex, as quantified in structural magnetic resonance imaging (MRI), and hypometabolism in posterior cingulate, parietal, temporal, and hippocampal regions, as measured by FDG-positron emission tomography (FDG-PET) [1]. Of note, the use of those procedures in AD clinical practice is relatively limited by invasiveness of the protocols or high-cost of instruments and exams.

The discovery of reliable blood biomarkers of AD would be a great improvement, as they are minimally invasive, potentially accessible everywhere, and intrinsically cost-effective. The current state-of-the-art in the field has been recently reviewed [2, 3]. Many different biological targets have been proposed as blood biomarkers of prodromal AD, as those based on the amyloid-β protein precursor (AβPP) processing, the molecules related to tangle pathology coming from tau dysregulation, markers

of neurodegeneration and microglia/astrocyte activation as neurofilament light (NF-L), neurogranin (Ng), sTREM2 and YKL-40, or AD-associated protein accumulation (for instance, α-synuclein and TDP-43), up to microRNA (miRNA) quantification [2–7]. Unfortunately, literature results are contradictory, probably because of a lack of standardization in assays and clinical inclusion criteria. In particular, many studies were centered on the comparison of healthy controls and AD patients, a choice that might be a confounding factor for diagnostic or prognostic purposes [8–13].

Clusterin (apolipoprotein J, CLU) has also been suggested as candidate plasma biomarker of AD, based on CLU gene involvement in AD risk and the availability of several association studies assessing CSF or plasma CLU level in prodromal dementia [14–18].

Keeping in mind the above scenario, it is critical to underscore that some differences in blood biomarkers between AD patients and age-matched healthy controls with normal cognition may be unspecific for disease neuropathology. In other words, those biomarkers might be sensitive not only to AD but also to other disorders inducing cognitive deficits in seniors. To account for this confounding variable, here we took advantage from the prospective, multi-centric clinical study named "IMI-PharmaCog-European ADNI" (http://www.pharmacog.org), where 144 aMCI patients were followed over time with the collection of clinical, neuropsychological, structural and functional MRI, electroencephalographic (rsEEG/ERP), CSF, and blood data. In the present study, we specifically tested the hypothesis that blood plasma measured molecules Aβ$_{42}$, Aβ$_{40}$, and CLU may be able to diagnose AD and monitor its progression (i.e., a period of 18 months) from prodromal disease stages.

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MATERIALS AND METHODS

Participant clinical features and classification

Participants' demographics, clinical, and neuropsychological data have been described in recent PharmaCog/E-ADNI studies. Briefly, 147 aMCI patients were enrolled in 13 European memory clinics of the Innovative Medicine Initiative (IMI) PharmaCog/E-ADNI project. The protocol of this study was designed in the framework of IMI and was aimed at improving the pathway of drug discovery in AD, with a main interest in disease-modifying drugs reducing Aβ$_{42}$ in the brain in AD patients at the prodromal stage of aMCI. Inclusion criteria were age between 55 and 90 years; complaints of memory loss; Mini-Mental State Examination (MMSE) score of ≥24; Clinical Dementia Rating score of 0.5; score on the logical memory test < 1 standard deviation from the age-adjusted mean; 15-item Geriatric Depression Scale score ≤5; and no neurologic, systemic or psychiatric comorbidity [19, 20]. We applied the diagnostic criteria for AD suggested by IWG-2 [1] and AA-NIH [21] guidelines. According to these guidelines, even at prodromal stage, AD is associated with 1) a reduction of CSF Aβ$_{42}$ and its increase at brain level and with 2) an increase of phospho-tau in both CSF and brain. IWG-2 and AA-NIH guidelines state that the diagnosis of AD can be done with Aβ$_{42}$ and tau biomarkers even with a single recording session, as AD is considered a progressive disease [1, 21]. Before study enrollment, each patient gave signed informed consent in compliance to the guidelines of local ethical committees. Data collected and generated have been always used in anonymous and aggregated form.

The aMCI patients were classified into two groups named "positive" (i.e., prodromal AD) and "negative" (i.e., stable aMCI) based on baseline CSF Aβ$_{42}$/P-tau levels as well as apolipoprotein E (APOE) ε4 genotype [22]. Specifically, aMCI patients were considered "positive" with CSF Aβ$_{42}$/P-tau levels lower than 15.2 for APOE ε4 carriers and lower than 8.9 for APOE ε4 non-carriers, otherwise "negative". These cut-offs were obtained by applying model-based classification methods (mixture models) [23] on baseline CSF Aβ$_{42}$/P-tau distribution, adjusted for APOE ε4 genotype.

Blood collection and plasma separation

All procedures involving patients were done after eligibility check according to inclusion criteria and informed consent signature. Blood for plasma preparation was collected by venipuncture at baseline, at month 12 and 18 during follow-up, resulting in a total of 3 venipuncture sessions.

Procedures for blood withdrawal and processing were standardized for all centers. Blood samples were processed within 1 h from the puncture.

Briefly, 10 mL of blood were collected into EDTA tubes of and centrifuged at 1600 g/4°C/15 min. The supernatant (plasma) was transferred into a new polypropylene tube after gentle shaking to avoid gradient effects and divided into aliquots of 250 μL in dry ice. Plasma was kept frozen at –80°C in temperature-monitored ultra-freezers (–80°C ± 5°C) until required.

Amyloid peptides 40 and 42 (Aβ$_{40}$, Aβ$_{42}$) and clusterin (CLU) ELISA determination

The assessment of plasma Aβ$_{42}$ and Aβ$_{40}$ was done with ELISA kits from Fujirebio (Fujirebio, Japan), namely Innotest β-amyloid(1-42) (code 81576), in presence of high-sensitivity secondary antibody conjugate (code 81587), and Innotest β-amyloid(1-40) (code 81585). The limit of detection (LOD) for the kits were 4.0 and 5.0 pg/mL, respectively. The assays dynamic ranges were 7.8–1000 pg/mL and 6.8–1000 pg/mL, respectively.

Human clusterin (apolipoprotein J) concentration in plasma was measured by an ELISA kit (BioVendor – Laboratorní medicína a.s., Czech Republic, code BV53031). The kit limit of detection (LOD), defined as concentration of analyte giving absorbance higher than mean absorbance of blank plus three standard deviations of the absorbance of blank, was 0.5 ng/ml. The assay dynamic range was from 5 to 160 ng/mL.

Statistical analysis

Statistics was done by SPSS software for descriptive statistics and R software (version 3.4.1) for the computational analysis based on Linear Mixed Models. The aMCI participants' features were compared by parametric Student's *t*-tests or non-parametric Mann-Whitney's U-test, depending on Gaussian distribution and using Chi-square tests for categorical data. Due to the exploratory nature of the present study, significance level was set at $p < 0.05$ [24].

Two different types of Linear Mixed Models (LMMs, performed by R-package lme4) for repeated measures were used with all available values of the plasma biomarkers (Aβ$_{42}$, Aβ$_{40}$, Aβ$_{42}$/Aβ$_{40}$, and CLU) and clinical variables. Random intercept and random slope were considered to account for individual differences at baseline as well as for individual change over follow-up. The output of the LMMs was presented in terms of standardized β coefficient, corresponding *p*-value and effect size (pseudo η^2) calculated as ratio of explained variability of interaction effect on total variability of each model.

In detail, a first group of LMMs was conducted to identify plasma measured molecules (dependent variable) that differently progressed in prodromal AD compared to stable aMCI patients in the whole aMCI group. This was performed by adding age, gender, education, time, group (corresponding to CSF status), time X group interaction as covariates. Only plasma measures with significant group X time interaction were of interest, meaning that they differently progressed over-time between groups. A second group of LMMs was conducted to evaluate the association between cognitive changes (ADAS-Cog 13, dependent variable) and peripheral plasma measured molecules, in the whole group and in prodromal AD patients only. This was performed by adding age, gender, time and biomarker as covariates. Plasma assessed molecules showing a significant effect of the biomarker factor were of interest, meaning that they were associated to cognitive decline.

RESULTS

Patients' features

In the IMI-PharmaCog/E-ADNI study, a cohort of 144 aMCI out of the 147 enrolled patients underwent CSF standard dementia biomarker evaluation (Aβ$_{42}$, T-tau, P-tau) and APOE genotyping. Table 1 summarizes IMI-PharmaCog/E-ADNI cohort demographic and clinical features. Due to plasma unavailability of some patients, the number of aMCI patients who were included for plasma measure assessment was lower (i.e., 128 aMCI patients). The main demographic and clinical characteristics of the included patients are reported in Table 2. In both Tables 1 and 2, after stratification according to baseline Aβ$_{42}$/P-tau ratio values in the CSF as a function of APOE genotype [22], the aMCI patients were classified as "positive" (prodromal AD) or "negative". We also statistically compared mean values reported in Table 2 to Table 1 in order to exclude a selection bias due to the unavailable samples in the plasma analysis. There were no differences between the "positive" (prodromal AD) and "negative" aMCI groups (data not shown).

Amyloid peptides 40 and 42 (Aβ$_{40}$, Aβ$_{42}$), clusterin (CLU), and prodromal AD

Figures 1 to 4 summarize the results of an exploratory statistical analysis about plasma Aβ$_{42}$,

Table 1
Clinical and socio-demographic features of amnesic mild cognitive impairment (aMCI) patients recruited for the IMI-PharmaCog/E-ADNI study. Patients were stratified as CSF $A\beta_{42}$/P-tau "positive" and "negative" according to APOE4-specific cut-offs [22]

	"negative" MCI ($n = 63$)	"positive" MCI ($n = 81$)	p^a
Age, mean (SD)	68.3 (8.4)	69.8 (6.3)	0.208
Sex, F/M, No.	36/27	46/35	1.000
Education, mean (SD)	10.0 (4.3)	11.1 (4.4)	0.115
APOE ε4 carriers, No. (%)	3 (5)	63 (78)	**<0.001**
MMSE, mean (SD)	27.1 (1.8)	26.2 (1.8)	**0.006**
ADAS-Cog13, mean (SD)[b,c]	19.1 (5.9)	21.6 (8.1)	0.052
CSF biomarkers, mean (SD, pg/mL)			
$A\beta_{42}$	949 (244)	495 (132)	**<0.001**
P-tau	47 (15)	84 (38)	**<0.001**
T-tau	301 (149)	614 (394)	**<0.001**

[a]Parametric *t*-test (or corresponding non-parametric Mann-Whitney) for continuous Gaussian (or non-Gaussian) distributed variables and Chi-square test for categorical data. [b]Range 0–85, with 0 as the best score. [c]Information was missing for 1 patient. ADAS-Cog13, Alzheimer Disease Assessment Scale-Cognitive Subscale, version 13; $A\beta_{42}$, amyloid-β 42; APOE, apolipoprotein E; CSF, cerebrospinal fluid; P-tau, tau phosphorylated at threonine 181; SD, standard deviation; T-tau, total tau.

Table 2
IMI-PharmaCog/E-ADNI study patients who underwent plasma assessment

	"negative" MCI ($n = 52$)	"positive" MCI ($n = 76$)	p^a
Age, mean (SD)	68.2 (8.4)	69.5 (5.9)	0.30
Sex, F/M, No.	26/26	43/33	0.46
Education, mean (SD)	10.0 (4.2)	11.2 (4.5)	0.13
APOE ε4 carriers, No. (%)	2 (3.8)	61 (80)	**<0.001**
MMSE, mean (SD)	27.0 (1.7)	26.2 (1.8)	**0.012**
ADAS-Cog13, mean (SD)[b]	18.8 (5.7)	21.6 (8.1)	**0.033**
CSF biomarkers, mean (SD, pg/mL)			
$A\beta_{42}$	930 (239)	499 (133)	**<0.001**
P-tau	46 (15)	84 (37)	**<0.001**
T-tau	295 (146)	619 (397)	**<0.001**

[a]Parametric *t*-test (or corresponding non-parametric Mann-Whitney) for continuous Gaussian (or non-Gaussian) distributed variables and by Chi-square test for categorical data. [b]Range 0–85, with 0 as the best score. ADAS-Cog13, Alzheimer Disease Assessment Scale-Cognitive Subscale, version 13; $A\beta_{42}$, amyloid-β 42; APOE, apolipoprotein E; CSF, cerebrospinal fluid; P-tau, tau phosphorylated at threonine 181; SD, standard deviation; T-tau, total tau.

$A\beta_{40}$, $A\beta_{42}$/$A\beta_{40}$ ratio, and CLU in the "positive" (prodromal AD) and "negative" aMCI groups at the three recording timepoints (T0, T12, and T18 months). The figures also show the same plasma measures in aMCI patients as a whole group. Exploratory univariate statistical tests compared the mean values between the groups or between timepoints ($p < 0.05$).

Figure 1 shows the results for plasma $A\beta_{42}$. There was no significant mean difference between the two aMCI groups at any time ($p > 0.05$). Furthermore, there was no significant mean difference among the three timepoints when all aMCI patients were considered as a whole group ($p > 0.05$).

Figure 2 plots the results for plasma $A\beta_{40}$. There was a marginal significance when comparing T0 level between the two aMCI groups ($p = 0.06$), with mean values slightly lower in the "positive" than the "negative" group. Furthermore, there was no significant mean difference among the three timepoints when all aMCI patients were considered together ($p > 0.05$).

Figure 3 illustrates the results for plasma $A\beta_{42}$/$A\beta_{40}$ ratio. There was no significant mean difference between the two aMCI groups at any time ($p > 0.05$). Moreover, there was no difference among the three timepoints when all aMCI patients were grouped.

Finally, Fig. 4 describes the results for CLU. There was no significant mean difference between the two aMCI groups at any time ($p > 0.05$). In contrast, CLU increased in all aMCI patients as a whole group over time, with a significant difference from T0 to both T12 and T18 ($p < 0.001$). This difference was common to the "negative" and "positive" aMCI groups.

To refine the above statistical analysis, we applied Linear Mixed Models to the plasma measures using the factors Group ("positive" and "negative" aMCI) and Time (T0, T12, and T18). Table 3 reports the proportion of variability in plasma measures over time explained by Time, Group (CSF status as defined by $A\beta_{42}$/P-tau), and Time X Group interaction. All plasma measures considered reported a significant effect of Time (for $A\beta_{42}$, $p < 0.001$; $A\beta_{40}$, $p = 0.009$; $A\beta_{42}$/$A\beta_{40}$ ratio, $p = 0.006$; CLU, $p < 001$), showing their changes over time (T0 to T18) regardless of the group. Conversely, none of those measures showed a significant "diagnostic" Group effect ($p > 0.05$).

Noteworthy, there was a significant Time X Group effect for plasma $A\beta_{42}$, showing that compared to the "negative" aMCI group, the "positive" (prodromal AD) aMCI group was characterized by a significant decrease of the measure over time ($p < 0.05$), in line with the feature of a disease progression biomarker.

Correlation of $A\beta_{40}$, $A\beta_{42}$, $A\beta_{42}$/$A\beta_{40}$, and clusterin (CLU) with ADAS-cog13 score

Table 4 reports the results of Linear Mixed Models testing the correlation over time of plasma measured molecules ($A\beta_{42}$, $A\beta_{40}$, $A\beta_{42}$/$A\beta_{40}$ ratio, and CLU)
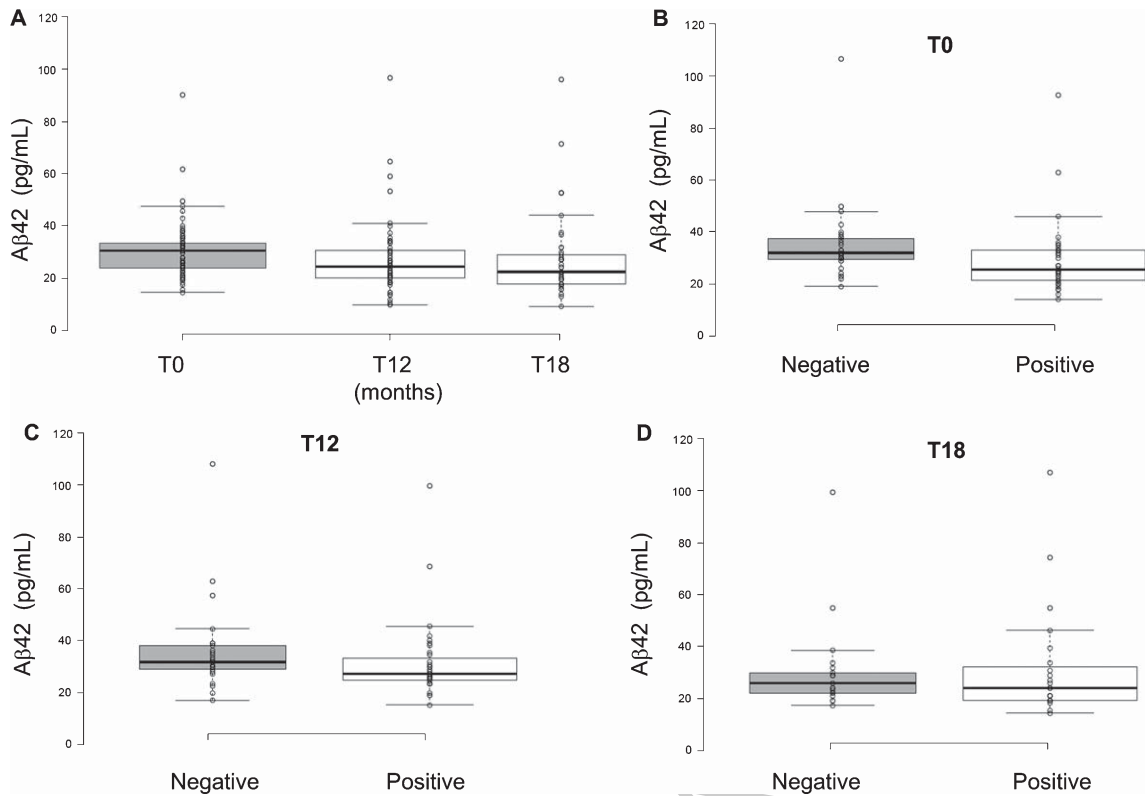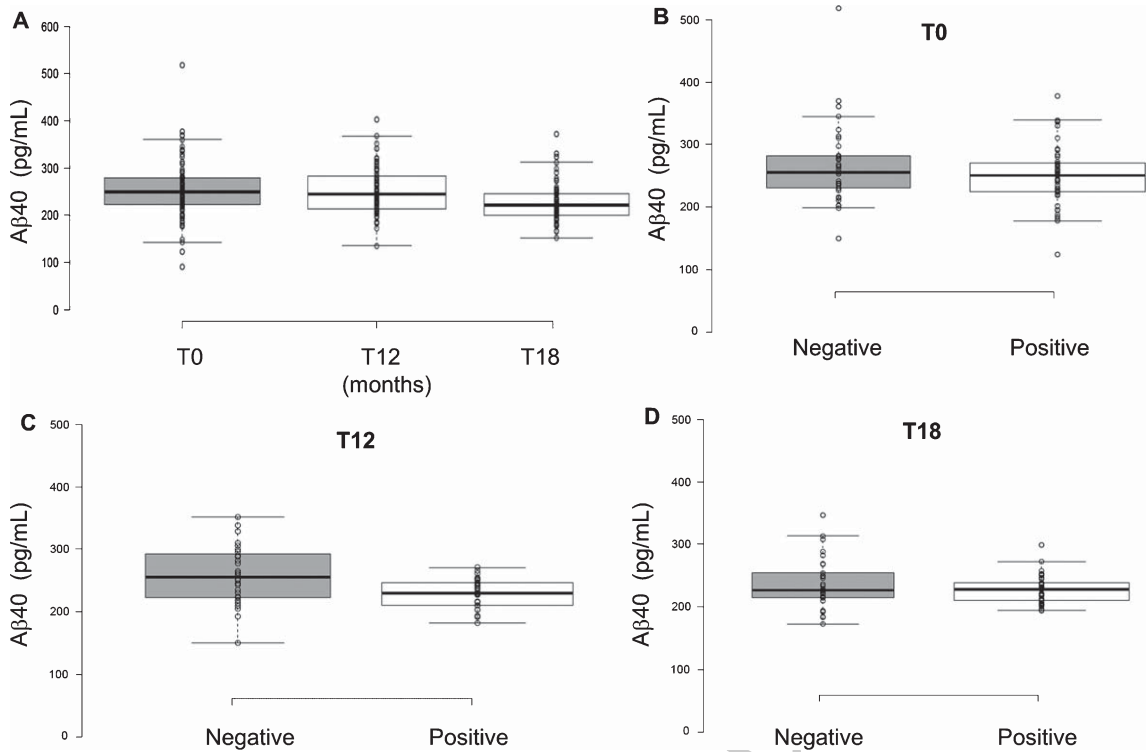
Fig. 1. Plasma Aβ$_{42}$ levels in the IMI-PharmaCog/E-ADNI study. (A) Whole aMCI group over time; (B) Baseline (T0); (C) Assessment after 12 months from T0 (T12); (D) Assessment 18 months from T0 (T18). Data are presented as box-plot, with the upper box line indicating the 3rd quartile, the lower one the 1st quartile and the bold line the median. The single measures are also indicated as empty circles. "Negative" and "positive" refer to the classification of aMCI according to the APOE-specific cut-offs [22].

with ADAS-Cog13 score. When all aMCI patients were considered as a whole, there was a significant association with ADAS-Cog13 score for all plasma measures ($p < 0.003$) with the only exception of Aβ$_{40}$. This association reflected the increase of ADAS-Cog13 scores over the follow-up period due to a progressive cognitive impairment of the whole population.

When the "positive" (prodromal AD) aMCI group was considered alone, there was still a significant association between plasma Aβ$_{42}$ ($p < 0.05$) and ADAS-Cog13 score, thus suggesting a clinical relevance of that measure. The same was true for plasma Aβ$_{42}$/Aβ$_{40}$ ratio ($p < 0.05$). Instead, no association was found for Aβ$_{40}$ alone or CLU ($p > 0.05$).

DISCUSSION

The IMI-PharmaCog/E-ADNI longitudinal study aimed at testing candidate biomarkers suitable to diagnose prodromal AD in aMCI patients and track disease progression over time (up to 24 months). As a novelty in the field of biomarker discovery for aMCI progressing to AD, to overcome the possible confounding effect of comparing healthy subjects to cognitively impaired patients, we used a control group with the same kind of amnesic deficits of the experimental group. Specifically, we compared blood plasma biomarkers in aMCI patients "positive" (i.e., prodromal AD) versus "negative" classified basing on their CSF Aβ$_{42}$/P-tau level and APOE ε4 carrier status [22]. In the present investigation, we tested the diagnostic or disease monitoring value of plasma Aβ$_{42}$, Aβ$_{40}$, and CLU in aMCI patients with probable prodromal AD. Among many other plasma biomarker candidates, the present ones have obvious links to AD pathogenic mechanisms and a direct counterpart on relevant CSF and PET diagnostic measures used in AD research.

However, the collected plasma and DNA samples may be suitable for other AD blood biomarker candidates of interest, including a variety of protein, lipid, and microRNA species, as well as mitochondrial

Fig. 2. Plasma $A\beta_{40}$ levels in the IMI-PharmaCog/E-ADNI study. (A) Whole aMCI group over time; (B) Baseline (T0); (C) Assessment 12 months from T0 (T12); (D) Assessment 18 months from T0 (T18). Data are presented as box-plots, with the upper line indicating the 3rd quartile, the lower one the 1st quartile and the bold line the median. The single measures are also indicated as empty circles. "Negative" and "positive" refer to the classification of aMCI according to the calculated algorithm, as reported above.

genes or DNA epigenetic modification patterns [25–32]. They may be evaluated in future studies carried out in PharmaCog/E-ADNI "positive" and "negative" aMCI groups.

Concerning the diagnostic value of the assessed blood biomarker candidates, the present results showed that plasma $A\beta_{42}$ was not specifically associated with the group of "positive" aMCI patients (prodromal AD) when the three recordings (baseline, 12, and 18 months) were considered as a whole. Furthermore, plasma $A\beta_{42}$, $A\beta_{42}/A\beta_{40}$ ratio, and CLU in all aMCI patients as a whole were correlated with cognitive status as measured by ADAS-Cog13 score, namely the neuropsychological procedure typically used in AD clinical trials [33, 34]. These findings suggest that plasma $A\beta_{42}$, $A\beta_{42}/A\beta_{40}$ ratio, and CLU are clinically relevant for aMCI cognitive status and may partially explain the variance of the results in previous studies where plasma $A\beta_{42}$ and $A\beta_{40}$ (or their ratio) were informative on AD status, especially when AD patients with dementia were compared to seniors with intact cognition [8]. Indeed, this association between plasma biomarkers and AD status was not always confirmed [11, 13]. So large variance of results in previous investigations might partially depend on cognitive status of participants in the AD and control groups as well as disease stage of AD participants. Of course, technical reasons may also contribute to the observed variance in previous findings [35, 36]. For example, the importance of plasma $A\beta_{42}$ as a biomarker of AD has been recently re-evaluated thanks to the contribution of Nakamura and colleagues, who measured plasma $A\beta_{42}$ with an advanced high-performance procedure based on immunoprecipitation followed by mass spectrometry [7]. In light of this improved protocol, they were able to demonstrate an interesting correlation between plasma $A\beta_{42}$ measurements and CSF and PET biomarker counterparts in AD patients [7]. In addition, Nabers and colleagues developed an immune-infrared sensor to measure the secondary structure change of all soluble Aβ peptides in human plasma that correlated to CSF AD biomarkers and amyloid PET in a cross-sectional study and was predictive of AD in a prospective cohort [37].
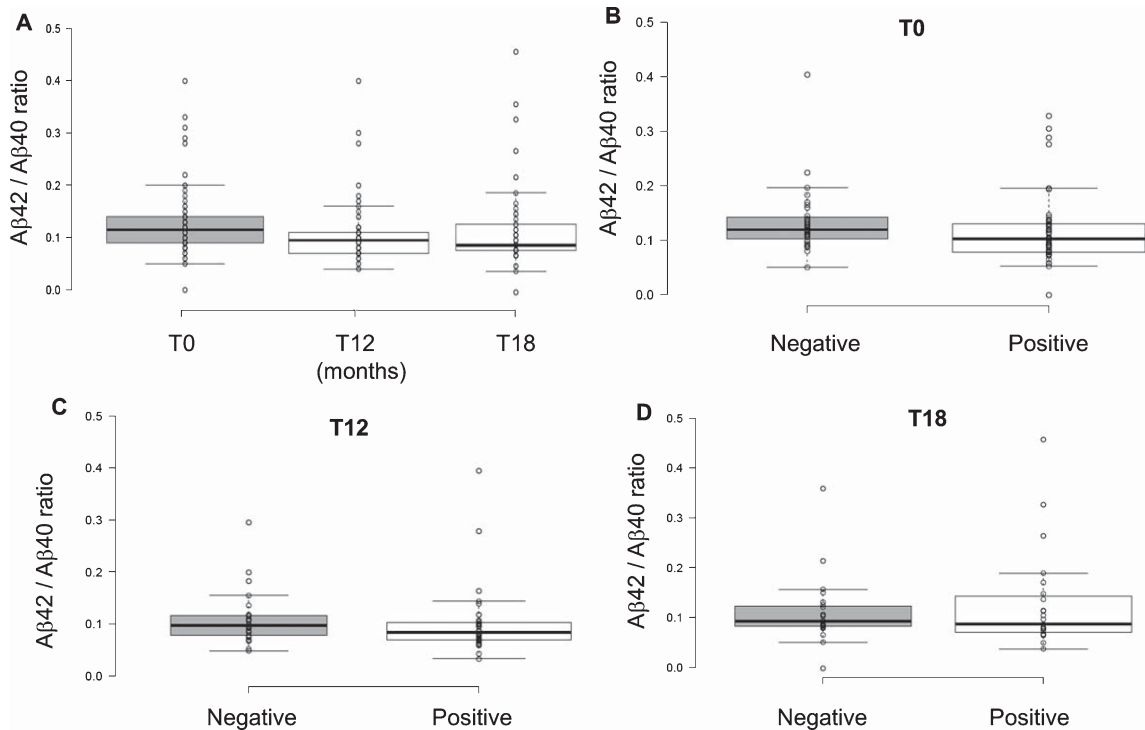
Fig. 3. Plasma $A\beta_{42}/A\beta_{40}$ ratios in the IMI-PharmaCog/E-ADNI study. (A) Whole aMCI group over time; (B) Baseline (T0); (C) Assessment 12 months from T0 (T12); (D) Assessment 18 months from T0 (T18). Data are presented as box-plots, with the upper line indicating the 3rd quartile, the lower one the 1st quartile and the bold line the median. The single measures are also indicated as empty circles. "Negative" and "positive" refer to the classification of aMCI as already described.

As for the informative value of the considered plasma biomarkers on prodromal AD progression, the present results show that plasma $A\beta_{42}$ was specifically associated with the "positive" aMCI group (prodromal AD) as a function of time (i.e., follow-ups at 12 and 18 months). The prodromal AD patients showed a specific significant decrease of plasma $A\beta_{42}$ over time, which correlated with the deterioration of cognitive performance as revealed by ADAS-cog13 scores. To our knowledge, this is the first demonstration that plasma $A\beta_{42}$ may be used as a biomarker of prodromal AD progression, taking into account the confounding variable of aMCI patients' cognitive status.

Available literature shows mixed results about the possible correlation between CSF and plasma $A\beta_{42}$. In our study, we checked for this correlation in "positive" aMCI subjects, finding no evidence of correlation (data not shown). Indeed, some previous studies failed in demonstrating a significant relationship [38, 39] while other were successful in finding a correlation, either positive [8] or negative [40]. Here we report that compared with the "negative" aMCI

subjects, the "positive" aMCI showed a steeper longitudinal lowering in the $A\beta_{42}$ at plasma level (interaction between Group x Time factors) but not a lowering considering all recording sessions as a whole (i.e., no Group factor effect). This outcome cannot be explained by an effect of different cognitive deficits in the experimental ("positive" MCI) and control ("negative" MCI) groups, as both were MCI (indeed, the condition of MCI might theoretically be due not only to AD neuropathology but also other parallel causes affecting cognitive functions, namely a cerebrovascular disease). A conclusive explanation of the above results requires further investigation. We can just speculate that plasma $A\beta_{42}$ may be influenced not only by the brain amyloidosis but also by the interaction between such process and others related to AD (e.g., tauopathy and neurodegeneration). However, any interpretation of the results should take into account that our study focused on a limited time of follow-up (i.e., until 18–24 months) and that CSF could be collected only at baseline and after 18 months. Therefore, our findings are a proof-of-concept to be cross-validated
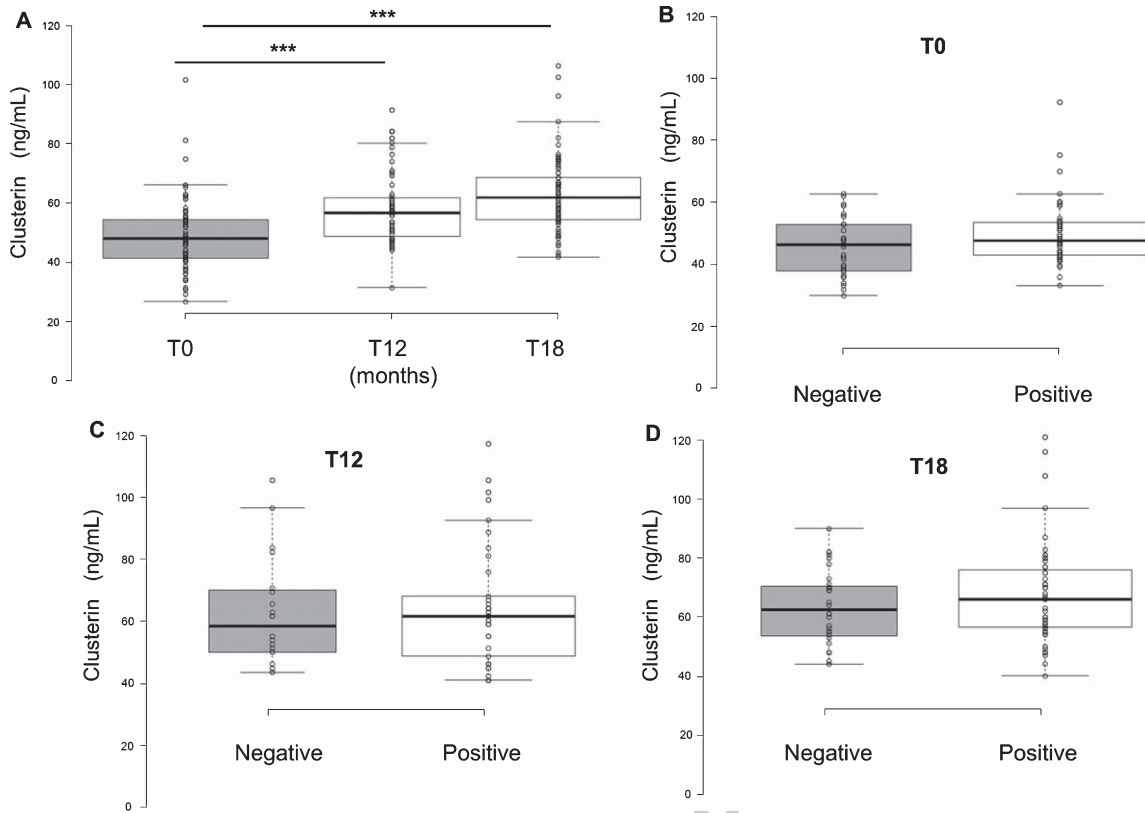
Fig. 4. Plasma clusterin (CLU) in the IMI-PharmaCog/E-ADNI study. (A) Whole aMCI group over time; (B) Baseline (T0); (C) Assessment 12 months from T0 (T12); (D) Assessment 18 months from T0 (T18). Data are presented as box-plots, with the upper line indicating the 3rd quartile, the lower one the 1st quartile and the bold line the median. The single measures are indicated as empty circles. For "negative" and "positive" aMCI classification, see above. ***$p < 0.001$ versus T0, ANOVA and Tukey's *post-hoc* test.

Table 3

Linear Mixed Models for the analysis of selected plasma molecules in aMCI patients stratified as "positive", as prodromal AD, and "negative" as a control group, according to cut-offs of CSF $A\beta_{42}$/P-tau [22]. The model included age, sex, baseline MMSE score, Time, Group ($A\beta_{42}$/P-tau status), and Time X Group interaction as predictors. Significant ($p < 0.05$) effects are shown in bold

Measure (dependent variable)	Time		Group		Time X Group		
	Std β	p	Std β	p	Std β	p	Pseudo η^2 (Effect size)
$A\beta_{42}$	0.209	**<0.0001**	0.011	0.937	0.151	**0.048**	0.25
$A\beta_{40}$	0.206	**0.009**	0.142	0.286	0.036	0.815	0.01
$A\beta_{42}/A\beta_{40}$	0.193	**0.0006**	0.062	0.725	0.147	0.326	0.01
CLU	0.462	**<0.001**	0.085	0.562	0.062	0.663	0.01

Std β, standardized β coefficient of Linear Mixed Model; CLU, clusterin (apolipoprotein J).

with a longitudinal study in which $A\beta_{42}$ in the CSF and plasma are systematically recorded in positive MCI subjects over time.

The second plasma biomarker investigated in the present study was clusterin (apolipoprotein J, CLU), based on the promising literature addressing the role of CLU in blood-based early AD diagnosis. In fact, it was reported that CLU levels are elevated in brain, CSF, and plasma of AD patients with dementia and MCI [41]. Moreover, CLU is functionally associated with amyloid species, and many genetic association studies have confirmed its role as a predisposing factor for AD [42–45]. Despite these considerations, we were unable to show a significant value of CLU neither in prodromal AD diagnosis nor in the disease progression. There was, however, a slight increase of plasma CLU over time both in "negative" and "positive" aMCI groups, suggesting that this blood biomarker may track the progression of brain disorders but not specifically for AD. It can be speculated

Table 4
Longitudinal Mixed Model Analysis of the association between cognitive decline
(ADAS-Cog 13, dependent variable) and peripheral circulating molecules in the
whole group and in the Aβ$_{42}$/P-tau positive MCI patients [22]. Significant
($p < 0.05$) effects are shown in bold

Measure (independent variable)	Whole MCI group		Aβ$_{42}$/P-tau positive MCI patients	
	Biomarker Standardized β	p	Biomarker Standardized β	p
Aβ$_{42}$	0.267	**0.003**	0.225	**0.046**
Aβ$_{40}$	0.047	0.346	0.079	0.150
Aβ$_{42}$/Aβ$_{40}$	0.225	**0.002**	0.226	**0.016**
CLU	0.149	**0.002**	0.096	0.092

CLU, clusterin (apolipoprotein J).

that this blood biomarker may have a slower variation with disease onset and progression in comparison to plasma Aβ$_{42}$, and increased amyloid burden may be required to reveal robust CLU differential expression in brain or in the periphery. In the present experimental design, the plasma follow-up time (18 months) may be too limited to conclusively demonstrate an AD-specific variation of CLU longitudinally.

In conclusion, we suggest that after the diagnosis of aMCI according to criteria based on CSF Aβ$_{42}$ lowering and P-tau increase [1, 21], also plasma Aβ$_{42}$ measured with standard ELISA procedure may be sensitive to prodromal AD progression and cognitive impairment. Instead, we did not confirm a diagnostic value of plasma Aβ$_{42}$, at least at that prodromal stage. We are confident that in a short-term period other studies may cross-validate our results, also taking advantage from recent technological advancements in the assessment of plasma Aβ$_{42}$ [7], and we propose to speed-up plasma Aβ$_{42}$ assay translation to clinical setting. Finally, our results on plasma Aβ$_{42}$ may be integrated by future studies that systematically investigate the relationship between CSF versus plasma phospho-tau and total tau, considering the remarkable steps forward in the measurement of those biomarkers [46].

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