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Plasma proteomics for the identification of Alzheimer's disease

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Abstract

Less invasive biomarkers for early Alzheimer's disease (AD) are urgently needed. The present study aimed to establish a panel of plasma proteins that accurately distinguishes early AD from physiological aging and to compare the findings with previous reports. Fifty eight healthy controls (CON) and 109 patients with AD dementia were randomly split into a training (40%) and a test (60%) sample. Significant proteins to differentiate between the CON and the AD dementia groups were identified in a comprehensive panel of 107 plasma analytes in the training sample; the accuracy in differentiating these two groups was explored in the test sample. A set of five plasma proteins was identified, which differentiated the CON vs. the AD dementia with a sensitivity of 89.36% and a specificity of 79.17%. A biological pathway analysis showed that four of the five proteins belonged to a common network with amyloid precursor protein and tau. Apolipoprotein E was the only protein that was both significant in the present report and in one previous proteomic study. The study provides a piece of evidence in support of the feasibility of a blood-based biomarker approach in AD diagnostics; however, further research is required because of issues with replicability.

Keywords

Alzheimer's disease; dementia; biomarker; early diagnosis; prognosis; proteomics

Introduction

Identifying Alzheimer's disease (AD) at an early clinical stage is of great interest, especially in view of treatment strategies aiming at disease modification and probably showing their greatest impact as long as symptoms are only minor. Therefore, biomarkers are urgently

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List of Supplemental Digital Content
Supplemental Digital Content 1.doc

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needed that can be applied in large-scale screening programs to accurately identify individuals with early AD dementia or memory-impaired older adults at risk of future cognitive decline and associated disability. Established biomarkers including the cerebrospinal fluid (CSF) proteins total-Tau (tTau), phosphorylated-Tau $(pTau)_{181}$ and Amyloid-β $(Aβ)_{1-42}$[1] as well as structural or functional imaging studies[2] show acceptable sensitivity and specificity for the diagnosis of early AD; furthermore, CSF and imaging research has identified some interesting new marker candidates such as CSF soluble amyloid precursor proteins (sAPPs)[3] and amyloid imaging.[4] However, these technology- and cost-intensive or invasive methods are not suitable for large-scale screening programs. As lumbar puncture is a relatively safe procedure that is well tolerated in most instances,[5] it still is an invasive procedure, which restricts its use to a limited number of indications. Hence, biomarkers need to be developed that can be obtained with relative ease from peripheral body fluids to replace or assist CSF biomarkers.

Blood plasma is an easily accessible body fluid that can be accessed with minimal discomfort to the patient, which facilitates the sampling of large cohorts and serial sampling. The plasma proteome holds great promise for the discovery of biomarkers for a range of diseases since it is in contact and molecular exchange with every organ and tissue including the brain and therefore reflects many physiological and pathophysiological changes.[6] Importantly for an AD biomarker, the brain uses signaling proteins found in blood to control many body functions, and central and peripheral inflammatory and immune mechanisms are linked to AD.[7] Changes in these signaling proteins associated with AD are likely to cause a disease-specific phenotype in blood.[8] The present study aimed to detect changes due to AD in a comprehensive panel of plasma proteins and to explore if the results of previous studies are replicable.

Methods

Study design and sample

The data used in this study were obtained from the Alzheimer's Neuroimaging Initiative database (ADNI; www.loni.ucla.edu/ADNI; see Supplemental Digital Content 1 for further details). On October 27, 2011, information from 566 subjects was available in the ADNI biomarker core database; complete data sets from 58 healthy elderly control subjects (CON), 380 patients with mild cognitive impairment (MCI), and 109 patients with AD dementia were used for the present study. Information from clinical follow-up visits six to 60 months after the baseline assessment was also available for the MCI group.

The study was approved by the institutional review boards of all participating centers and written informed consent was obtained from all participants or authorized representatives after extensive description of the ADNI according to the 1975 Declaration of Helsinki. The study is registered at ClinicalTrials.gov (registration number NCT00106899, http://clinicaltrials.gov).

Protein assays

Fasting 500 μl EDTA plasma samples were obtained from the participants in the morning and put into the freezer within 120 minutes in most cases; aliquoting and processing was conducted according to ADNI standardized operating procedures. A 190 analytes multiplex immunoassay panel, referred to as the human discovery map, was developed for the Luminex xMAP platform (Luminex Corp., Austin, TX, USA) by Rules-Based Medicine (RBM Inc., Austin, TX, USA). This assay was designed to contain proteins previously associated with cancer, cardiovascular disease, metabolic disorders, and inflammation; in addition plasma proteins believed to be involved in AD-associated cell signaling were

included.[8] The analysis of plasma samples on the human discovery map was conducted centrally at the facilities of RBM. The detailed quality control procedures and results are available from the ADNI website (http://adni.loni.ucla.edu/2010/11/new-set-of-proteomics-data-will-be-available-friday-november-19th/). Data of 146 analytes which had passed the strict ADNI quality control was used for the present study (see Supplemental Digital Content 1 for a complete listing).

Baseline CSF samples were obtained from the study participants and analyzed at the ADNI biomarker core laboratory at University of Pennsylvania; the detailed sampling methods have been described previously.[9] The CSF concentrations of $A\beta_{1-42}$, tTau, and $pTau_{181}$ were measured using the multiplex xMAP Luminex platform with Innogenetics immunoassay kit-based reagents (INNO-BIA AlzBio 3; Ghent, Belgium).

Statistical analysis

All statistical analyses were performed using SPSS, v19.0 (IBM corp., Somers, NY, USA) and R-Software, v2.13.0 with the Q-Value package (http://genomics.princeton.edu/storeylab/qvalue/).[10] 39 of the 146 RBM analytes that had passed quality control were excluded from the present analyses because they had more than 1% missing data, resulting in a final set of 107 plasma proteins. Prior to the actual statistical analyses, all 107 plasma analytes were assessed for normal distribution within each diagnostic group using Kolmogorov-Smirnov-tests. The false discovery rate (FDR),[11] which controls the expected proportion of incorrectly rejected null hypotheses (type-I errors), was used to account for the issue of multiple comparisons, i.e. results at $q < 0.05$ were regarded significant.

A cross-validation study design was used to identify the significant plasma proteins, which is a common procedure in clinical proteomics.[12,13] The current study population was randomly divided into one training sample and one test sample including 60% (N_{CON}=34; $N_{AD\ dementia}$ = 62) and 40% (N_{CON} = 24; $N_{AD\ dementia}$ = 47) of the population, respectively. In the training sample, independent Student's t-test or Mann-Whitney U-test was used, as appropriate, in order to compare the RBM analyte concentrations between the CON vs. the AD dementia groups. Subsequently, the significant proteins were used as independent variables in a logistic regression analysis (LR) with stepwise forward variable selection with diagnostic status as the dependent variable (CON vs. AD dementia), followed by a receiver operating characteristic curve (ROC) analysis. The same LR model with subsequent ROC analysis was then applied to the validation sample to confirm the results of the test sample. In order to compare the accuracy of the new plasma biomarkers with established AD markers, the concentrations of $A\beta_{1-42}$, tTau and $pTau_{181}$ were tested in a separate LR model in the test sample. Non-parametric correlations (Spearman's rank coefficient) were calculated in the entire study sample in order to explore the associations between each individual pair of significant blood biomarkers and between the blood and the CSF markers. In addition, Cox proportional hazard models were applied to assess the ability of baseline biomarker levels to predict the progression from MCI to AD dementia. Data from patients who did not convert during up to 5 years follow-up period were statistically censored at the date of the last assessment.

Pathway Analysis

The computational gene network prediction tool Ingenuity Pathway Analysis (IPA), v8.5 (Ingenuity System, Redwood City, CA, USA) was used to analyze the biological connections of the significant plasma proteins among each other as well as their probable relation to AD pathology. For this purpose, the protein symbol identifications were first translated to gene symbols (Supplemental Digital Content 1) and subsequently fed into the biological pathway analysis. In addition, a likelihood score was computed for each network,

with a score of 3 indicating that there is only a 1 in 1000 chance that the assembly of a set of focus genes in a network could be explained by random chance alone.

Comparison with previous plasma proteomic studies

In order to explore if AD is associated with a replicable blood proteomic signature, thirteen previously published independent plasma proteomic studies were identified in Medline, which reported significant differences between AD dementia and physiological aging.[8,14–25] These studies reported a total of 79 significant plasma proteins, 62 of which had been found in a single study, whereas the remaining 17 were significant in at least two independent cohorts (see Supplemental Digital Content 3 for a complete listing). The overlap between the findings of the previous reports with the results of the present study was examined; furthermore, the 16 proteins with significant group differences in at least two previous cohorts were subjected to the above detailed statistical procedures.

Results

Characteristics of the study sample are presented in Table 1. In the training set, a total of seven analyzed plasma proteins showed significant differences after FDR correction in the comparison between the CON group vs. the AD dementia group, including alpha-1-microglobulin (A1M; q = 0.03), apolipoprotein E (ApoE; q = 0.03), brain natriuretic peptide (BNP; q = 0.04), betacellulin (BTC; q = 0.02), Eotaxin-3 (q = 0.02), interleukin-16 (IL16; q = 0.03), and serum glutamic oxaloacetic transaminase (SGOT; q = 0.02). These seven analytes were therefore used as predictors in the subsequent stepwise LR models, in which five of these seven proteins contributed significantly to the differentiation of CON vs. AD dementia in the training sample, including A1M ($p < 0.01$), ApoE ($p = 0.001$), BNP ($p < 0.01$), IL16 ($p = 0.0001$), and SGOT ($p = 0.02$). The LR model including the five significant proteins (LR_{Plasma}) had a sensitivity of 87.10% and a specificity of 79.41%. Applied to the validation sample, the LR_{Plasma} model showed comparable results with a sensitivity of 89.36% and a specificity of 79.17%. In comparison, the regression model restricted to the established CSF biomarkers $A\beta_{1\text{-}42}$, tTau, and $pTau_{181}$ (LR_{CSF}) resulted in a sensitivity of 97.78% and a specificity of 91.30% for the differentiation between CON vs. AD dementia in the test sample (Table 2).

During a maximum of 5 years follow-up, 163 patients with MCI progressed to AD dementia (mean follow-up time 2.77 ± 1.00 years), whereas 217 remained in the MCI stage (mean follow-up time 2.50 ± 1.10 years). In the Cox regression model with stepwise forward variable selection entering the five identified plasma proteins as predictors, only ApoE was significant with lower concentrations in the progressive MCI group (hazard ratio 0.34; $p = 0.01$).

The correlation analysis revealed significant associations between the individual blood biomarkers as well as between the blood and the CSF markers. Significant results were found for correlations between A1M with BNP ($r = 0.31$, $p < 0.001$) and IL16 ($r = 0.24$, $p = 0.001$), as well as BNP with SGOT ($r = -0.18$, $p = 0.02$). $A\beta_{1\text{-}42}$ was correlated with all of the tested blood biomarkers (A1M: $r = -0.23$, $p < 0.01$; ApoE: $r = 0.43$, $p < 0.001$; BNP: $r = -0.21$, $p < 0.01$; IL16: $r = 0.25$, $p = 0.001$; SGOT: $r = 0.26$, $p = 0.001$). Furthermore, tTau was correlated with A1M ($r = 0.29$, $p < 0.01$) and IL16 ($r = -0.25$, $p = 0.01$), and pTAU was correlated with A1M ($r = 0.24$, $p < 0.01$), ApoE ($r = -0.20$, $p = 0.01$), BNP ($r = 0.17$, $p = 0.03$), and IL16 ($r = -0.24$, $p < 0.01$).

To further understand the biological relevance of the tested biomarkers for AD, the potential signaling pathway within the final five RBM analytes and APP as well as tau was explored using the computational gene network prediction tool IPA. This analysis identified a

regulatory network connecting four signaling genes related to four plasma proteins (A1M, ApoE, BNP, and IL16) with APP and tau (Supplemental Digital Content 1), which had a significant likelihood score of 17.

Regarding the comparison of the present results with the findings of previous proteomic studies, 30 out of 79 previously described plasma proteins were also available in ADNI; only ApoE was significant both in the present and in one previous report.[14] In addition, eleven out of 17 plasma proteins with significant concentration differences between AD dementia and physiological aging in at least two previous studies could be tested in the present analysis. However, none of these eleven proteins contributed to the differentiation between the AD dementia and CON groups in the ADNI cohort (results not shown).

Discussion

A blood-based biomarker panel for the early identification of AD is highly desirable due to a number of considerations. The present study reports data from the ADNI including a set of 107 plasma analytes; we found a plasma proteomic signature of the early clinical stages of AD that offered a degree of diagnostic accuracy close to the established CSF biomarkers. However, the missing overlap with the results of previously published comparable studies dampens our enthusiasm.

Given that AD is a biologically complex neurodegenerative disease that is unlikely to be caused by a single pathogenic event (or cascade of events), the finding of a panel of plasma biomarkers characterizing AD rather than a single marker was an expected result.[26] The present study addressed a central shortcoming of the available AD biomarkers, namely the need for less invasive procedures for ascertainment. $A\beta_{1\text{-}42}$ and $tTau/pTau_{181}$ in CSF are important components of the clinical diagnostic process of AD;[27] however, none of them is exclusive for AD dementia.[1,28–30] Another potential limitation of these established CSF markers is that they only mirror two, albeit central, aspects of the multifactorial nature of AD. Therefore, a more comprehensive set of markers covering different aspects of the disease is likely to contribute relevant information that may improve diagnostic performance. In addition to these biological considerations, practical issues related to CSF sampling hinder the wider use of biomarkers in CSF. In the present study, a set of five plasma proteins was found that significantly differed between the early clinical stages of AD and physiological aging. A regulatory network analysis showed that four of these markers, A1M, ApoE, BNP, and IL16 were closely linked to APP and tau signaling; this finding was also supported by the additional correlation analyses. ApoE shows strong genetic association with AD.[31] IL16 is a chemo-attractant for certain immune cells, and its upregulation has been observed in various cerebral pathologies[32,33] including AD.[34] BNP is a circulating hormone that has been connected to dementia, partly in conjunction with cardiovascular disease.[35] A1M is a ubiquitous plasma and tissue protein that acts as heme-scavenger and thereby protects against reactive oxidative stress,[36] potentially preventing Aβ aggregation.[37] Biological functions related to this network include cellular death, lipid metabolism, and molecular transport, all functions that are involved in neurodegeneration and AD pathogenesis.[38] In addition to this regulatory network, SGOT was also found to be altered in AD in the present study. SGOT is a biomarker of peripheral inflammation such as in hepatitis,[39] muscle damage,[40] and myocardial infarction[41]; SGOT is also an important enzyme in aminoacid metabolism and a common clinical measure for liver function.[42] Altered SGOT enzyme activity in AD prefrontal cortex has been reported before.[43] A previous study of our group suggested that SGOT levels in CSF might improve the diagnosis of AD dementia in combination with tau.[44] The relationship between AD and peripheral inflammation is yet unclear[45] and further research is warranted to elucidate this

association. To sum up these considerations, the biomarker panel in our present study includes proteins in blood that seem to reflect the heterogeneous pathomechanisms of AD.

Compared with two previous analyses of the ADNI dataset,[46,47] using different statistical approaches from the one applied in the present study, an overlap between significant blood proteins was only found for ApoE, which was also significant in a number of other previous studies. In our present study, ApoE contributed both to the differentiation between AD dementia and physiological aging and to the prediction of cognitive decline in MCI. The lacking prediction of cognitive decline in MCI by the other markers may possibly be explained by changing protein concentrations with disease progression. ApoE is a small transport protein highly expressed in liver and brain. In brain, ApoE is well-known as an amyloid chaperone protein associated with AD neuropathology.[48] It may have a role in preventing Aβ oligomerization and amyloidogenesis and ApoE protein concentration in blood correlates with brain amyloid burden in cognitively normal individuals.[26] However, reports on ApoE blood concentrations in AD dementia are conflicting, showing unchanged [49], increased [50], or decreased levels,[51,52] the latter being in line with our present findings. Decreased plasma ApoE levels may contribute to a disrupted cerebral lipid metabolism. In addition, lower peripheral ApoE levels may also reduce the efficiency of Aβ peptide clearance.[48] It is important to note that while *APOE* genotype is a well-established AD risk factor, the association between *APOE* variations and blood ApoE protein levels is less clear.[53]

The large number of discovery phase proteomic studies is in contrast to the limited number of validation studies, which highlights the challenges of replication.[54, 55] Problems may arise from different experimental designs and analytical methods as well as heterogeneous cohorts.[56] However, the diagnostic relevance of findings without replication is highly questionable. In the present work, the combination of five plasma proteins resulted in a reasonably good differentiation between patients with AD dementia vs. healthy control subjects, but four of these proteins had not been reported before. Furthermore, only ApoE was identified in one of two previous analyses of the ADNI dataset using different statistical approaches.[57,58] In summary, the present findings indicate that the blood proteome is a promising source for reliable AD biomarkers but also that replicable findings are crucial.

Limitations of our study include the lack of histopathological confirmation of AD diagnoses; however, the validity of the clinical diagnoses at specialized centers has repeatedly been confirmed by autopsy series.[59] The ADNI cohort may not truly represent the whole population with AD and future studies will have to show if comprehensive biomarker panels are also able to distinguish between certain AD sub-types, e.g. those with a relevant vascular or inflammatory component. Most importantly, future studies will need to replicate our findings and also to relate the plasma biomarker panel to core pathological features of AD such as MRI hippocampal atrophy or in-vivo amyloid burden using positron-emission-tomography. Even though a comprehensive set of blood proteins was selected according to a hypothesis-driven approach, other involved proteins might have been missed. The cross-validation approach used in our study is widely applied in discovery phase clinical proteomic studies. However, findings without replication in truly independent cohorts have to be handled carefully. Discrimination of patients and controls using the set of proteins identified in the present study is likely to be less accurate in independent cohorts.

To conclude, our study presents a further piece of evidence for the existence of a specific blood-based proteomic profile of AD, which may be of interest for diagnostic purposes. However, more research is urgently needed to identify reliable blood-based biomarkers and our study also highlights the dire need for replication of discovery phase findings. Peripheral markers of disease identified in proteomic or genomic studies are influenced by a number of

internal and external factors such as gender,[60] age, concomitant diseases,[61] and medications.[62] Future research will have to carefully adjust for these nuisance factors.

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Table 1

Characteristics of the study sample

	CON	AD dementia	MCI
N[#]	58	109	380
Age, years	75.14 (5.75)	74.68 (8.06)	74.81 (7.39)
MMSE, points	28.93 (1.15)	23.53 (1.91) *	27.01 (1.77) *
Education, years	15.60 (2.70)	15.17 (3.21)	15.64 (3.05)
Sex, M/F	28/30	62/47	244/136
BMI	27.15 (4.00)	25.68 (3.80)	26.05 (3.99)
ApoE ε4, % carrier	8.62	69.72*	53.95
$A\beta_{1\text{-}42}$, pg/mL (CSF)	250.62 (21.61)	143.02 (39.44) *	163.48 (53.44) *
tTAU, pg/mL (CSF)	63.59 (21.38)	117.91 (55.99) *	100.92 (54.69) *
$pTAU_{181}$, pg/mL (CSF)	20.39 (7.33)	40.83 (17.74) *	34.88 (16.90) *
A1M, μg/mL (Plasma)	1.04 (0.11)	1.12 (0.10) *	1.08 (0.12) *
ApoE, μg/mL (Plasma)	1.86 (0.15)	1.71 (0.20) *	1.68 (0.18) *
BNP, pg/mL (Plasma)	2.76 (0.39)	3.04 (0.33) *	3.02 (0.32) *
IL16, pg/mL (Plasma)	2.61 (0.12)	2.54 (0.13) *	2.54 (0.17) *
SGOT, μg/mL (Plasma)	3.64 (1.01)	2.96 (0.81) *	3.69 (2.00)

Data presented as mean (SD) where appropriate;

* significant differences compared with the CON group at $p<0.05$.

CON: cognitively normal controls; MCI: mild cognitive impairment; AD: Alzheimer's disease; MMSE: Mini-Mental-State Examination; ApoE: Apolipoprotein E; BMI: body mass index; $A\beta_{1\text{-}42}$: Amyloid-$\beta_{1\text{-}42}$; tTau: total-Tau; $pTau_{181}$: phosphorylated-Tau_{181}; CSF: cerebrospinal fluid; A1M: Alpha-1-Microglobulin; ApoE: Apolipoprotein E; BNP: Brain Natriuretic Peptide; IL16: Interleukin-16; SGOT: Serum Glutamic Oxaloacetic Transaminase.

[#] N for the sample with CSF results: CON 58, MCI 193, AD dementia 100.

Table 2

Performance of the biomarker sets in the differentiation between healthy controls vs. patients with AD dementia

	LR_{CSF}	LR_{Plasma}
ROC AUC	0.98	0.93
SN (%)	97.78	89.36
SP (%)	91.30	79.17
ACC (%)	95.59	85.92
PPV (%)	95.65	89.36
NPV (%)	95.45	79.17

ROC: receiver operating characteristic; AUC: area under the curve; SN: sensitivity; SP: specificity; ACC: classification accuracy; PPV: positive predictive value; NPV: negative predictive value; LR_{CSF}: logistic regression model with the 3 CSF markers $A\beta_{1\text{-}42}$, tTau, and $pTau_{181}$ as the independent variables; LR_{Plasma}: logistic regression model with the combination of 5 selected plasma proteins as the independent variables.