Several Direct and Calculated Biomarkers from the Amyloid-β Pool in Blood are Associated with an Increased Likelihood of Suffering from Mild Cognitive Impairment

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Abstract. Validation of cost-effective, non-invasive methods to identify early (pre-clinical) Alzheimer’s disease (AD) is increasingly becoming a key research challenge. We have developed two ELISA sandwich colorimetric tests for the accurate detection of amyloid-β (Aβ)1-40 and Aβ1-42: i) directly accessible (DA) in the plasma, ii) recovered from the plasma sample (RP) after diluting the plasma sample in a formulated buffer, and iii) associated with the remaining cellular pellet (CP). These tests were carried out on samples from healthy controls (n = 19) and individuals with mild cognitive impairment (MCI; n = 27) with amnestic-hippocampal syndrome to investigate whether this comprehensive approach may help to explain the association between blood Aβ levels and MCI. A logistic regression analysis detected seven direct or calculated markers (CP 40, DA 42, RP 42, DA/CP 40, DA/RP 42, DA/CP 42, and DA 42/40) with significant odds ratios (OR) after they were dichotomized with regard to the median of the pooled population. In particular, the likelihood [OR (95% CI)] of having MCI for patients with catCP 40, catDA/RP 42, catDA/CP 42, or catDA 42/40 below the corresponding population median (“positive test”) was 11.48 (1.87–70.52), 22.09 (3.19–152.61), 11.48 (1.87–70.50), and 9.54 (1.77–51.38)-fold higher, respectively, than in those with a “negative test” after adjusting for the effect of the ApoE genotype. These results are congruent with the hypothesis that changes in blood Aβ levels may be associated with the initial stages of AD. Thus, these Aβ blood biomarkers might be useful tools for screening for those at increased risk of developing AD.

Keywords: Aging, Alzheimer’s disease, diagnosis, ELISA

INTRODUCTION

Alzheimer’s disease (AD) is associated with extracellular deposition of amyloid peptides (Aβ), as well as intracellular accumulation of neurofibrillary tangles. Previously, the presence of amyloid and tau pathology in autopsied brains from cognitively healthy people was thought to refute the amyloid cascade hypothesis.
showed statistically significant associations between AD pathology and detectable signs of disease onset [6, 11–17].

Indeed, the majority of previous cross-sectional studies to an amyloid cortical pathology that heralds AD. Evidence that changes in brain Aβ levels due to an amyloid cortical pathology that heralds AD. This interpretation of AD’s natural history has driven recent research efforts toward developing earlier-course and preventive treatments and the search for surrogate diagnostic biomarkers [8].

In line with this interpretation, the work carried out in large multicenter initiatives such as the Alzheimer’s Disease Neuroimaging Initiative (ADNI), the Australian Imaging Biomarkers and Lifestyle Flagship Study of Ageing (AIBL), European-ADNI (E-ADNI), and many others has led to the proposal of a model in which the most widely validated biomarkers (MRI, PIB-PET, FDG-PET, and CSF levels of Aβ, tau, and phosphorylated-tau) become abnormal in an ordered sequence that parallels the hypothetical pathophysiological sequence of AD [9, 10].

However, these well-validated biomarkers are hampered by practical pitfalls that severely limit their application in large populations. The feasibility of these biomarkers for screening the general population once a preventive treatment has been developed also remains questionable. Consequently, there is increasing interest in the development of blood-based biomarkers among which Aβ peptides have attracted particular attention because of their proven mechanistic relationship with AD pathology and accumulating evidence that changes in brain Aβ are among the first detectable signs of disease onset [6, 11–17].

Our hypothesis is that changes in blood Aβ levels may reflect changes in brain Aβ levels due to an amyloid cortical pathology that heralds AD. Indeed, the majority of previous cross-sectional studies showed statistically significant associations between Aβ plasma markers (primarily Aβ40, Aβ42, or the Aβ42/Aβ40 ratio) and a determined diagnosis [healthy control (HC), mild cognitive impairment (MCI) or AD] [18–31]. However, the details of this association remain far from clear and the literature has produced controversial results (for a recent review, see [32]).

There are numerous confounding factors from a variety of sources that blur the presumptive association of Aβ blood levels and the diagnosis of AD. These include technical issues, the long pre-clinical course of the disease, and the variable patient history of the elderly, which can affect the metabolism of Aβ in different ways [33]. The interaction of all (or several) of these confounding factors could lead to conflicting—even entirely opposite—results coming from different studies.

Another relevant source of confusion is the fragmentary knowledge of the biology of Aβ in the blood. An important point to be stressed is that Aβ peptides in blood can be found free in the plasma, bound to plasma proteins, and bound to blood cells [34–39]. Therefore, a complete Aβ blood test should include the determination of peptide levels in each of these fractions. Our study represents a first step in this direction and an attempt to gain as much information as possible regarding the distribution of these peptides in the blood. To these ends, we separately assessed the peptide directly accessible in the plasma, the peptide that can be recovered after diluting the plasma sample in a formulated buffer, and the peptide that remains adhered to the cellular pellet after plasma collection.

The aim of the present study was to investigate whether this comprehensive approach could help explain the association between blood Aβ levels and the early stages of AD.

MATERIAL AND METHODS

The demographic characteristics of the participants are summarized in Table 1. The HC (n = 19) and MCI (n = 27) diagnostic groups were established according to the routines of the Memory Clinic of Fundació ACE as described elsewhere [40]. The MCI patients fulfilled the Mayo Clinic criteria with a clinical dementia ratio (CDR) of 0.5 and a normal MMSE. The patients were described as amnesic with hippocampal syndrome based on the Wechsler Memory Scale-III (WMS-III) as described elsewhere [41]. The MCI group was subdivided into those with a negative neuropsychological diagnosis (MCI-NId; n = 12) suggestive of a more advanced disease stage and those with a positive neuropsychological diagnosis (MCI-Nlp; n = 15) suggestive of a more advanced disease stage. To be classified as MCI-Nlp, patients present ≥4 points bilaterally on the Scheltens scale for medial temporal atrophy, as assessed using MRI, and no signs of hypometabolism in the medial temporal or cingular posterior region, as assessed using FDG-PET. To be classified as MCI-Nld, patients present ≥4 points bilaterally on the Scheltens scale for medial temporal atrophy, as assessed using MRI, and no signs of hypometabolism in the medial temporal or cingular posterior regions [42].

Written informed consent was obtained from every participant. The study protocols were reviewed and approved by the Ethical Committee of the Hospital Clínic i Provincial (Barcelona, Spain). Education level was recorded in five categories depending on the number of years of education. However, cases had to be...
regrouped into two categories (more or less than 8 years of education) to fulfill the lowest expected frequency condition in the contingency table.

**Blood sampling and biochemical determinations**

Blood samples from each participant were drawn in the morning after an overnight fast and were collected in polypropylene vials with EDTA and a protease inhibitor cocktail (Complete Mini, Roche Madrid Spain). The samples were immediately cooled to 4°C until processing which occurred in the first 24 hours after collection. The blood samples were centrifuged and both the plasma and the cell pellet were divided into aliquots and stored in polypropylene tubes at −80°C until analyzed. At no time was the material thawed or refrozen.

All samples were analyzed in triplicate in the same run for each of the three blood fractions using two specific ELISA sandwich kits, ABtest 40 and ABtest 42 (Araclon Biotech Ltd. Zaragoza, Spain), as described elsewhere [30]. Before analysis, plasma and blood cell samples were pretreated using dilution in a formulated saline buffer with 1% blocking polymer according to the supplier’s instructions.

We carried out three counts for both the \(A/\beta_40\) and \(A/\beta_42\) peptides in each blood sample. One count was performed using the undiluted plasma sample, another using the plasma sample diluted 1:3 with the aforementioned formulated buffer, and a third using the cellular pellet that remained after plasma collection. The peptide amount in the undiluted plasma sample corresponds to the directly accessible (DA) peptide. The 1:3 dilution of the plasma was chosen because it provided the maximum peptide recovery from the sample (see Supplementary Figure 1, available online: http://dx.doi.org/10.3233/JAD-121744). Thus, this count included the DA peptide and the peptide that was recovered from the plasma matrix (RP). Additionally, the peptide associated with the cellular pellet (CP) was measured in a 1:5 dilution of the pellet that remained after plasma collection. The sum of these three amounts is described as the \(A/\beta\) pool in blood (PIB) for either \(A/\beta_40\) or \(A/\beta_42\). Additionally, from these directly measured markers, we obtain the ratios of DA/RP, DA/CP, and RP/CP for each peptide and the ratios of DA \(A/\beta_42\) to DA \(A/\beta_40\), RP \(A/\beta_42\) to RP \(A/\beta_40\), and CP \(A/\beta_42\) to CP \(A/\beta_40\) (CP42/40) (see Table 2).

The inter-assay coefficients of variation (CV), as determined by the comparison of the same plasma control samples measured in the assay for either the diluted plasma, undiluted plasma or cells were 4.94% and 11.11% in ABtest40 and ABtest42, respectively. The limit of quantification (LQ) was 4.70 pg/ml for ABtest40 and 5.71 pg/ml for ABtest42. None of the

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### Table 2

**Direct and calculated \(A/\beta\) blood markers**

<table>
<thead>
<tr>
<th>Groups</th>
<th>n</th>
<th>DA 40 (pg/mL)</th>
<th>RP 40 (pg/mL)</th>
<th>CB 40* (pg/mL)</th>
<th>DA 42* (pg/mL)</th>
<th>RP 42* (pg/mL)</th>
<th>CB 42 (pg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HC</td>
<td>55.58 (14.62)</td>
<td>30.56 (7.08)</td>
<td>61.47 (67.64)</td>
<td>1.72 (0.39)</td>
<td>0.50 (0.31)</td>
<td>0.13 (0.06)</td>
<td>0.23 (0.16)</td>
</tr>
<tr>
<td>MCI</td>
<td>56.95 (18.08)</td>
<td>34.17 (8.46)</td>
<td>56.11 (14.71)</td>
<td>1.55 (0.35)</td>
<td>0.64 (0.17)</td>
<td>0.24 (0.07)</td>
<td>0.20 (0.11)</td>
</tr>
<tr>
<td>DA/ RP</td>
<td>40</td>
<td>D A/ B 40*</td>
<td>R P/ B 40</td>
<td>D A/ B 42**</td>
<td>R P/ B 42**</td>
<td>R P/ B 42**</td>
<td></td>
</tr>
<tr>
<td>HC</td>
<td>0.21 (0.06)</td>
<td>0.79 (0.30)</td>
<td>1.62 (0.98)</td>
<td>146.22 (12.68)</td>
<td>151.21 (66.22)</td>
<td>298.31 (82.57)</td>
<td></td>
</tr>
<tr>
<td>MCI</td>
<td>0.15 (0.09)</td>
<td>0.99 (0.74)</td>
<td>2.89 (2.23)</td>
<td>151.41 (29.47)</td>
<td>206.03 (126.69)</td>
<td>357.41 (149.86)</td>
<td></td>
</tr>
</tbody>
</table>

Data [median (interquartile range)] are in pg/mL. *p < 0.05; **p < 0.01; ***p < 0.001 in HC versus MCI groups.
determinations for ABtest40 was under its LQ, but 13% of the determinations of DA Aβ42 (although detectable) were under the LQ of ABtest42.

APOE genotyping was performed as previously described [43], using the amplification of genomic DNA, digestion with HhaI, and further analysis of the restriction fragments.

Statistical analysis

To compare demographic data between the two groups, we ran a Mann-Whitney U-test for continuous variables. Pearson’s chi-square test was used for the categorical variables. Binary logistic regression was performed to assess whether the level of chosen markers (split in two by the median of the pooled population) were associated with an increased likelihood of an MCI diagnosis in an unadjusted model and in models adjusted for age, ApoE genotype, or education level. Regarding their distribution within a group, only the markers with a p value <0.05 (Mann-Whitney test) were analyzed using logistic regression. Four individuals had outlier values for some of the markers, and 13% (six individuals) of the DA Aβ42 measurements were below the LQ. Because the primary results were obtained from the logistic regression analyses of dichotomous variables, the influence of these extreme data was considered irrelevant and they were not excluded from the study. Sensitivity and specificity of the Aβ markers and the hematological and blood biochemical variables was explored using a Spearman test. The SPSS v.20 software was used for statistical analysis. Graphics and figures were created using Graph Pad Prism v5.0.

RESULTS

The two primary diagnostic groups (HC and MCI) differed significantly depending on age, presence of ApoE e4, and education level (Table 1). By contrast, the two MCI subgroups (MCI-NIn and MCI-Nlp) were completely homogeneous regarding those variables.

A group comparison revealed that nine direct and calculated markers differed significantly between HC and MCI (Table 2). Interestingly, whereas the levels of DA 42 were lower in MCI than in HC, the levels of RP 42 and CP 42 were lower in HC than in MCI (Fig. 1). The most significant results in the comparison between HC and MCI were the DA/RP 42 ratio followed by the DA/CP 42 and DA 42/40 ratios, which were 48%, 45%, and 28% lower in the MCI group than in the HC group, respectively.

Additionally, seven of those nine markers (CP 40, DA 42, RP 42, DA/RP 42, DA/CP 42, DA 42/40, and RP 42/40) were found to differ significantly (p value equals 0.014, 0.008, 0.002, <0.001, 0.025, 0.001, 0.001, respectively) when the MCI-NIn group was compared with the HC group. By contrast, no marker displayed significant differences between the two MCI subgroups (MCI-NIn versus MCI-Nlp).

The binary logistic regression analysis showed that, once transformed in categorical (cat) variables that
between CP 42 and serum albumin (\(t\)ary Table 1). More significant correlations occurred for catDA/CP 42, and 0.75 for catDA 42/40, which are under the curve (AUC) was 0.80 for catDA/RP 42, 0.74 corresponding ROC curves (Table 4). In particular, the area markers were tentatively assessed using their corre-
sponded for. Thus, the likelihood of being an MCI OR when each demographic co-variable was com-
mits for. Thus, the likelihood of being an MCI marker’s level was equal or below the population median. *\(p<0.05\), **\(p<0.01\), ***\(p<0.001\).

Table 3

<table>
<thead>
<tr>
<th>MCI versus HC</th>
<th>OR</th>
<th>95% CI</th>
<th>OR</th>
<th>95% CI</th>
<th>OR</th>
<th>95% CI</th>
<th>OR</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>catCP 40 (^\ddagger)</td>
<td>5.60**</td>
<td>1.53</td>
<td>20.49</td>
<td>5.19*</td>
<td>1.27</td>
<td>21.20</td>
<td>11.48**</td>
<td>1.87</td>
</tr>
<tr>
<td>catDA 42 (^\ddagger)</td>
<td>5.60**</td>
<td>1.53</td>
<td>20.49</td>
<td>10.12**</td>
<td>1.91</td>
<td>53.55</td>
<td>3.52</td>
<td>0.72</td>
</tr>
<tr>
<td>catRP 42 (^\ddagger)</td>
<td>5.60**</td>
<td>1.53</td>
<td>20.49</td>
<td>15.31**</td>
<td>2.44</td>
<td>96.04</td>
<td>11.48**</td>
<td>1.87</td>
</tr>
<tr>
<td>catCP/CP 40</td>
<td>3.86*</td>
<td>1.06</td>
<td>12.77</td>
<td>2.57</td>
<td>0.67</td>
<td>9.81</td>
<td>5.41*</td>
<td>1.08</td>
</tr>
<tr>
<td>catDA/RP 42 (^\ddagger)</td>
<td>15.23**</td>
<td>3.38</td>
<td>68.55</td>
<td>669.88**</td>
<td>7.81</td>
<td>57434.87</td>
<td>22.09**</td>
<td>3.19</td>
</tr>
<tr>
<td>catDA/CP 42 (^\ddagger)</td>
<td>5.60**</td>
<td>1.53</td>
<td>20.49</td>
<td>7.21**</td>
<td>1.61</td>
<td>32.36</td>
<td>11.48**</td>
<td>1.87</td>
</tr>
<tr>
<td>catRP 42/40</td>
<td>8.90**</td>
<td>2.24</td>
<td>35.33</td>
<td>11.88**</td>
<td>2.34</td>
<td>60.30</td>
<td>9.54**</td>
<td>1.77</td>
</tr>
<tr>
<td>catRP 42/40</td>
<td>2.40</td>
<td>0.74</td>
<td>8.34</td>
<td>2.79</td>
<td>0.72</td>
<td>10.67</td>
<td>2.89</td>
<td>0.64</td>
</tr>
<tr>
<td>catRP 42/40</td>
<td>2.40</td>
<td>0.60</td>
<td>6.58</td>
<td>2.27</td>
<td>0.60</td>
<td>8.62</td>
<td>3.45</td>
<td>0.71</td>
</tr>
</tbody>
</table>

Table 4

<table>
<thead>
<tr>
<th>HC versus MCI</th>
<th>AUC</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>Test of equality</th>
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</thead>
<tbody>
<tr>
<td>CP 40</td>
<td>&lt;0.0083</td>
<td>0.737</td>
<td>0.741</td>
<td>0.717*</td>
</tr>
<tr>
<td>DA 42</td>
<td>&lt;0.001</td>
<td>0.789</td>
<td>0.667</td>
<td>0.704*</td>
</tr>
<tr>
<td>RP 42</td>
<td>&gt;0.0007</td>
<td>0.593</td>
<td>0.842</td>
<td>0.712*</td>
</tr>
<tr>
<td>DA/CP 40</td>
<td>&gt;0.0007</td>
<td>0.582</td>
<td>0.526</td>
<td>0.602*</td>
</tr>
<tr>
<td>DA/RP 42</td>
<td>&lt;0.0004</td>
<td>0.905</td>
<td>0.741</td>
<td>0.801**</td>
</tr>
<tr>
<td>DA/CP 42</td>
<td>&lt;0.0002</td>
<td>0.502</td>
<td>0.630</td>
<td>0.743**</td>
</tr>
<tr>
<td>DA 42/40</td>
<td>&gt;0.0007</td>
<td>0.947</td>
<td>0.630</td>
<td>0.754**</td>
</tr>
</tbody>
</table>

DISCUSSION

In the present work, we found nine markers from the A\(\beta\) pool in blood that differed significantly between a group of MCI patients of the amnestic-hippocampic type and a HC group. Once they were transformed in categorical variables, four of these markers (CP 40, DA/CP 42, DA/RP 42, and DA 42/40), presented sig-
ificant ORs even when the logistic regression model was adjusted for each of the relevant demographic co-
variables. The results showed an association beyond what could be attributable to pure chance between these A\(\beta\) blood markers and an MCI diagnosis. However, the significance of this association remains controversial, and there is a wealth of literature showing contradictory results (for recent reviews, see [28, 32, 44]). Nevertheless, apart from other relevant markers, our study revealed that levels of DA 42 and the ratio of DA 42/40 were lower in the MCI group than in the HC group, which is congruent with numer-
ous previous reports [18, 23, 25, 26, 28, 29, 45–48]. Furthermore, whereas DA 42 was lower in MCI than in HC, levels of RP 42 trended in the opposite direction. This finding could help to explain the variability of results obtained with the different assays used in vari-
ous studies. It is well known that A\(\beta\) peptides bind to plasma proteins and blood cells in amounts that depend on various factors that may or may not be related to AD pathology [34–38, 49–52]. Depending on the ability of a given assay to measure jointly the levels of peptide free in the plasma and a variable proportion of the A\(\beta\) peptides bound to plasma proteins, a single A\(\beta\) plasma measurement would fluctuate in one or other direction.
Concerning this point, our results suggest that separately quantifying the levels of Aβ peptides directly accessible in the plasma, the levels recovered from masking interactions with the plasma matrix and the levels associated to the cellular pellet could lead to a more comprehensive assessment of Aβ levels associated to the cellular pellet could lead to masking interactions with the plasma matrix and the accessible in the plasma, the levels recovered from /H9252
diagnostic ability of the Aβ among these three blood compartments, improving the better between plasma Aβ1–42 levels and brain amyloid deposits, thereby confirming results from other studies [60, 61]. Recently, other authors have reported correlations between levels of Aβ1–40 and Aβ1–42 free in plasma with the CSF Aβ1–42/Aβ42 ratio [38]. Interestingly, a recent publication from the Dominantly Inherited Alzheimer Network (DIAN) has shown that plasma Aβ1–42 levels are significantly higher in carriers of mutations for autosomal dominant AD than in non-carriers five years before such differences reach statistical significance in Aβ1–42 CSF levels [62].

Taken together, these results have boosted the interest in blood-based biomarkers and both Aβ1–40 and Aβ1–42 are increasingly considered moderate risk markers for AD well suited to be used as pharmacodynamic markers and eventually as a minimally invasive screen to identify people at increased risk of developing AD. Ongoing longitudinal studies may validate these results and confirm these hypotheses.

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SUPPLEMENTARY MATERIAL

Supplementary material can be found here: http://dx.doi.org/10.3233/JAD-121744

REFERENCES


nificant correlation between plasma and CSF Aβ1–42 levels in a sample of 715 ADNI subjects (205 HC, 348 MCI, and 162 AD). This correlation was slightly better between plasma Aβ1–42 levels and brain amyloid deposits, thereby confirming results from other studies [60, 61]. Recently, other authors have reported correlations between levels of Aβ1–40 and Aβ1–42 free in plasma with the CSF Aβ1–42/Aβ42 ratio [38]. Interestingly, a recent publication from the Dominantly Inherited Alzheimer Network (DIAN) has shown that plasma Aβ1–42 levels are significantly higher in carriers of mutations for autosomal dominant AD than in non-carriers five years before such differences reach statistical significance in Aβ1–42 CSF levels [62].

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