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Amyloid β peptides in plasma in early diagnosis of Alzheimer's disease: a multicenter study with multiplexing

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Abstract

We measured concentrations of Aβ peptides 1-42 and 1-40, and their ratio in plasma of patients carefully categorized clinically and neurochemically as having AD or other dementias with a newly commercially available multiplexing assay, characterized by reasonable laboratory performance (intraassay imprecision in the range of 1.3 - 3.8% for Aβ1-42, and 1.8 - 4.1% for Aβ1-40, interassay imprecision for Aβ1-42, Aβ1-40, and Aβ1-42/Aβ1-42 concentration ratio in the range of 2.3 -11.5%, 2.2 – 10.4% and 4.2 - 9.7%, respectively). Patients with AD or mild cognitive impairment of AD type (MCI-AD) whose clinical diagnosis was supported with CSF biomarkers (n=193) had significantly lower Aβ1-42 plasma concentrations (p<0.007), and Aβ1-42/1-40 ratios (p<0.003) compared to patients with other dementias and MCI of other types (n=64). No significant differences between persons with MCI of AD type and patients with early AD were observed, or between MCI of other types versus patients with early dementia of other types. Our findings reconfirm the hypothesis that alterations of biomarker concentrations occur early in a preclinical AD stage and that these alterations are also reflected in plasma.

Key words: amyloid β, Alzheimer's disease, multiplexing, dementia, biomarker

Introduction
The age-related surge in the number of people with dementia poses urgent public health challenges worldwide (Ferri, et al., 2005, Ott, et al., 1998). In particular, improvements in early detection and differential diagnosis of more common dementias such as Alzheimer’s disease (AD) are sorely needed, along with effective disease-modifying treatments. The availability of robust, validated biomarkers is considered to be one of the essential means to achieve these ends. For AD, such diagnostic markers should not only be quantifiable and reliable, but reflect the central pathogenic processes associated with the disease, namely the degeneration of neurons and their synapses as reflected in their defining characteristic lesions—senile plaques and neurofibrillary tangles (The Working Group on: "Molecular and Biochemical Markers of Alzheimer's Disease", 1998).

Although cerebrospinal fluid (CSF) biomarker studies based on lumbar puncture have been associated with very low rates of complications (Blennow, et al., 1993), such a technique is more invasive, and it poses a practical and psychological barrier to its use as a general screening tool. Alternatively, several studies suggest that concentration of Aβ in plasma may help in the early diagnosis of AD, but results seem conflicting (Freeman, et al., 2007, Graff-Radford, et al., 2007, van Oijen, et al., 2006). These differences may, in part, be related to the use of suboptimally standardized in-house tests. To circumvent this confounding issue, we measured plasma Aβ concentrations in clinically and neurochemically characterized groups using a new standardized multiplexing approach for the first time.

Materials and methods

Patients

The study was approved by the ethics committees of all the participating universities, and all patients and/or their relatives gave their informed consent. Fourteen German psychiatric university departments participated in the project (http://www.kompetenznetz-demenzen.de). 2113 subjects passed the screening examinations and
were assigned to one of the relevant diagnostic groups. There were 1870 patients (790 dementia, 1080 MCI patients) and 243 control subjects (201 healthy, 42 disease controls).

Dementia cases were further subdivided into AD (n = 577), frontotemporal dementia (FTD, n = 51), vascular dementia (VD, n = 35), mixed AD/VD (n = 72), Lewy body dementia (n = 8), Parkinson dementia (n = 5), Huntington’s disease and corticobasal degeneration (n = 2 each), progressive supranuclear palsy and multisystem atrophy (n = 1 each), other (n = 11) and other or undetermined (n = 25). Patients were diagnosed according to standard criteria: For AD, ICD-10 and NINCDS/ADRDA criteria were applied, and for MCI, a broad definition was used, including decline of cognitive ability (more than one SD) in at least one of the following domains: verbal learning and memory, nonverbal learning and memory, word fluency, naming, visuoconstruction, cognitive speed or executive function, as evidenced by standardized tests. There could be minor changes in complex activities of daily living (∑ B- ADL < 4). A CDR score of 0.5 was applied as a further criterion. Based on the clinical presentation, MCI patients received an additional clinical label of "presumable pre-AD", "presumable pre-mixed-dementia", "pre-FTD", "pre-VD" etc., or "unclassified". From these cases, 599 CSF samples were acquired (298 of MCI, 259 of dementia, 42 of disease controls) (Kornhuber, et al., 2009).

For this study, the total cohort of patients was divided into two groups according to neurochemical findings in the CSF (Table 1): the first group with pathologic results of neurochemical dementia diagnosis (NDD+; n=216) characterized by a decreased Aβ42/Aβ40 CSF concentration ratio (Aβ ratio, cut-off, 0.11 (Lewczuk, et al., 2004)) accompanied by an increased CSF concentration of phospho-Tau181 (pTau181; cut-off 70 pg/mL); and the second group with normal results of neurochemical dementia diagnosis (NDD-; n=135) characterized by a normal Aβ ratio (i.e. higher that 0.11) accompanied by normal pTau181 levels (below 50 pg/mL). Patients with 'borderline' CSF pTau181 concentration (50-70 26pg/mL) were not included.
Furthermore, a subgroup of 257 patients was subdivided into four groups based on clinical diagnoses supported by corresponding CSF-based neurochemical data: (1) the group of patients with early Alzheimer's dementia and pathologic NDD findings (D-AD/NDD+, n=109), (2) patients with other early dementias and normal NDD findings (D-O/NDD-, n=13), (3) patients with MCI of AD type and pathologic NDD findings (MCI-AD/NDD+, n=84), and (4) patients with MCI of non-Alzheimer type with normal NDD results (MCI-O/NDD-, n=51).

Cognitive dysfunction was assessed with the CERAD neuropsychological test battery (Thalman, et al., 1998), memory impairment was confirmed by deficits in WMS-R Logical Memory (Härting, et al., 2000). Functional decline, prerequisite for AD diagnosis, was assessed using the Bayer-ADL scale (Hindmarch, et al., 1998). A diagnosis of MCI corresponded to a CDR score of 0.5, while a diagnosis of mild dementia corresponded to a CDR score of 1.0 or 0.5, respectively. Severity of dementia was graded according to the Mini Mental State Examination (Folstein, et al., 1975). Cognitive impairment was not strictly expected to be exclusively in the memory domain, which is in line with the newer definitions of MCI, like those proposed by Petersen (Petersen, 2004), and Winblad et al. (Winblad, et al., 2004). In our project, the term "MCI-AD" was only assigned if: (a) test results documented some objective deficits, (b) the clinical CDR rating, derived from all available information and integrating all information, was 0.5, and (c) other explanations than AD seemed unlikely after the very detailed and thorough work-up. Certainly, the early sign of AD most often is "amnestic MCI". In some cases, however, the leading feature of incipient AD can be a "posterior" disorder, with apraxic deficits and visuoconstructive deficits, and some unusual AD cases begin with language deficits. The neurophysiologists of our multicenter project were trained to recognize these unusual cases.

Collecting of blood and CSF
Material (CSF and EDTA-blood) was collected according to the protocols described elsewhere (Lewczuk, et al., 2006). Briefly, lumbar puncture was performed with the patient in the sitting position. In addition to collection of the CSF for routine diagnosis (2-5 mL), an additionally 4.5 mL CSF was also drawn into the same polypropylene test tube. The tube was gently shaken, and the CSF was centrifuged immediately after collection (1600 g, room temperature, 15 min) and then aliquoted into 16 polypropylene test tubes (250 µL each), and frozen within 30-40 min after the puncture. Blood was collected from all subjects by venipuncture into a standard monovette containing EDTA (Saerstedt, Nümbrecht, Germany). Immediately after the collection, the blood was centrifuged at 1600 g to generate plasma. The material was at no time thawed/refrozen. All the aliquots of frozen samples were shipped on dry ice to one center (University of Erlangen), where subsequent analyses were performed.

**Assays**

ELISAs and APOE genotyping were performed as described elsewhere (Lewczuk, et al., 2008). Determination of Aβ peptides in plasma was performed using the INNO-BIA plasma Aβ forms multiplexing assay (Innogenetics, Gent, Belgium). This is a multiplex microsphere-based Luminex xMAP technique in which the beads are coated with the C-terminal-specific antibodies: 21F12 and 2G3, binding Aβ42 and Aβ40, respectively. A non-Aβ-binding antibody is added to account for the plasma matrix interference such as heterophilic antibodies. For detection, a biotynilated antibody is used specifically recognizing the N-terminus of Aβ peptides (3D6). The resulting fluorescence signal was read with a Luminex 100 IS analyzer.

To test intra-assay imprecision, five human plasma samples, collected and stored identically as all other study samples, were assayed 9-10 times in an experiment that was repeated twice. To test for interassay imprecision, five control samples provided with the assays were tested in 6-13 experiments conducted on different days.
2 Statistical analysis

The difference in plasma ratio Aβ1-42/Aβ1-40 between NDD+ and NDD- subjects was tested by T-test assuming equal or unequal variances as appropriate. For the three outcomes, the subgroup analyses were performed in a general linear model framework. The model contained all subgroups (combinations of AD status, NDD status, and dementia group). Effects of dementia were addressed with an omnibus Wald test. In the simplified model, the groups for which the NDD status is consistent with the clinical diagnosis were compared by a simple contrast. Between-center heterogeneity was addressed by including random center effects in the mixed model. For all analyses, normality assumptions were verified.

Results

Analytical performance of the Aβ multiplexing assay

Coefficients of variation of intra-assay imprecision were in the range of 1.3 - 3.8% for Aβ1-42, and in the range of 1.8 - 4.1% for Aβ1-40. Coefficients of variation of inter-assay imprecision were in the range of 2.3 - 11.5% for Aβ1-42, and in the range of 2.2 - 10.4% for Aβ1-40. Coefficients of variation of inter-assay imprecision of Aβ1-42/1-40 concentration ratio were in the range of 4.2 - 9.7%.

Aβ peptides in patients' plasma

We observed highly significant differences in Aβ1-42/1-40 plasma concentration ratios between the groups of patients with abnormal (NDD+) versus normal (NDD-) CSF biomarker profiles (Figure 1; T-test assuming equal variances, p<0.001).
Plasma Aβ1-42 concentrations, Aβ1-40 concentrations, as well as Aβ1-42/1-40 ratios in the four groups of patients, whose clinical diagnoses were supported by corresponding CSF findings, are presented in Figure 2 (A, B, and C, respectively).

Since there were no statistically significant differences in Aβ1-42/1-40 ratio between MCI stage and dementia stage in either 'AD-NDD+' or 'Other dementias-NDD-' groups, respectively (Wald test in linear model, p=0.94 for Aβ1-42; p=0.42 for Aβ1-40; and p=0.94 for the Aβ1-42/1-40 ratio), we combined 'MCI' and 'Dementia' stages for 'AD' and 'Other Dementia' groups, respectively, for statistical analysis. There was a statistically significant difference with respect to Aβ1-42 concentration (Figure 2A; Wald test in linear model, p<0.007) and Aβ1-42/Aβ1-40 ratio (Figure 2C; Wald test in linear model, p<0.003) between these groups. However, we did not observe a significant difference in the Aβ1-40 plasma concentrations between the groups (Figure 2B; Wald test in linear model, p=0.51). We observed a very weak correlation between Aβ1-42 in plasma and in the CSF (R=0.21, p<0.01, assuming linear correlation; data not shown).

Discussion

The main outcome of our study was the highly significant decreases in plasma Aβ1-42 concentrations and Aβ1-42/Aβ1-40 ratios in patients with early AD or MCI of the AD type compared to patients with other dementia types. Patients were categorized according to a rigorous protocol: in the fourteen German gerontopsychiatric university departments well-defined clinical and neuropsychological criteria were used (http://www.kompetenznetzdemenzen.de), and the neurochemical diagnostic procedures were defined in Erlangen (Lewczuk, et al., 2004, Lewczuk, et al., 2004, Lewczuk, et al., 2008).

Categorization of dementia patients is still an unresolved problem, and even postmortem analysis must be considered carefully for two reasons: a) neither Aβ plaques nor
Tau/pTau tangles are pathognomonic for Alzheimer's disease, and b) there is usually substantial time difference between the diagnostic lumbar puncture and the autopsy, and hence neuropathology does not necessarily reflects the stage of the disease when the CSF (or plasma) biomarkers were analyzed. To try to minimize this issue, we used two independent diagnostic tools: clinical/neuropsychological supported by neuroimaging, and neurochemical (CSF analysis of five biomarkers: Ab1-42, Abx-42, Abx-42/x-40 ratio, total Tau, and 7pTau181), and only patients fulfilling both criteria were considered for the study. In addition, we assumed that if our categorization strategy is correct, the resulting AD cohort should be enriched with APOE ε4 careers, which turned out to be the case. This rigorous diagnostic protocol hopefully limited the number of falsely grouped patients; however, we are aware of the fact that it cannot completely eliminate the discussed patients' categorization issue.

While the decrease in plasma Ab1-42/Ab1-40 ratio was observed between AD and other dementia in this study, a similar decrease in Ab42/Ab40 ratio using different antibodies in a similar assays were observed in other prospective studies (Graff-Radford, et al., 2007, van Oijen, et al., 2006), although in a previously published report, Hansson and colleagues did not find significant alterations in Ab42 plasma concentration (Hansson, et al., 2008).

Moreover, in agreement with the CSF results of the Blennow's group (Hansson, et al., 2006, Zetterberg, et al., 2003), as well as with our own studies on different biomarkers involved in Aβ metabolism (Lewczuk, et al., 2008), we did not observe differences between biomarkers concentration/ratio between MCI stage and early dementia, which may reconfirm the hypothesis that the alteration of biomarkers concentrations might be observed many years before the onset of clinical symptoms of dementia. All these observations further strengthen the idea that blood Aβ concentrations in AD patients might be affected, although it must be stressed that it is too early to clearly predict their role as diagnostic tools. Furthermore, confirmation of these findings were certainly attractive for early, i.e. pre-clinical, diagnosis of dementia, especially as soon as the first AD treatment or prophylaxis are on the market.
Moreover, currently the sensitivity and specificity of the blood based neurochemical dementia diagnostics (NDD) does not reach the performance of CSF based NDD (Wiltfang, et al., 2005).

Inconsistencies between plasma Aβ studies have often been related to pre-analytical factors, therefore in the current study the plasma (EDTA-blood) and CSF were collected according to rigorously defined standard operating procedures (Lewczuk, et al., 2006). Furthermore, reproducibility and repeatability of the new Aβ assay were tested prior to performing the study, and intra- and inter-assay imprecision of less than 12% were reported, which is in agreement with the data on analytical performance of this assay as reported by an independent group (Hansson, et al., 2008). Such levels of reproducibility are close to those reported using different technologies but the same antibodies (Gelfanova, et al., 2007). Thus in conjunction with additional technologies, such as Western blot (Bibl, et al., 2007) or quantitative mass spectrometry (Gelfanova, et al., 2007), this high-precision multiparameter x-MAP™ technology could be further employed to understand relationships between different pools of Aβ and AD pathological mechanisms.

The 10% to 15% reduction of Aβ1-42 and Aβ1-42/Aβ1-40 ratio is in line with the 30%-50% reduction in CSF (for a recent review see (Lewczuk and Wiltfang, 2008)). However, the weak correlations between plasma Aβ measurements and CSF found in this study or absence of such correlation as reported by other groups (Mehta, et al., 2000, Vanderstichele, et al., 2000) indicate that the mechanisms of changes in plasma and brain are complex. Indeed, although the brain is regarded as the primary source of Aβ in plasma, other sources, such as e.g. platelets and mononuclear blood cells, need to be taken into account (Di Luca, et al., 1998, Maler, et al., 2008), as well as the effects of transport across the blood-brain barrier and the different clearance mechanisms in blood and CSF (Bell, et al., 2007). One of the limitations of our study is certainly that we did not analyze healthy control subjects, however, in the study from the group of Blennow (Hansson, et al., 2008) with the
In conclusion, with the availability of the new, well-standardized, and commercially available multiplexing assay to measure Aβ peptides, plasma Aβ forms can be reliably measured, which opens the way for more studies to determine whether blood-based biomarkers can be used at baseline to predict eventual progression to AD in individuals entering memory clinics with early symptoms of dementia. Currently, however, it is still difficult to predict if analysis of the biomarkers in blood can be of diagnostic utility, and the analysis of the biomarkers in the CSF remains, to our opinion, state of the art tool of neurochemical dementia diagnostics.
Acknowledgements

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1 Figures legend

2 Fig 1. Comparison of Aβ1-42/1-40 concentration ratios in patients with normal (NDD negative) and abnormal (NDD positive) biomarkers in the CSF. Horizontal bars represent averages.

5 Fig. 2A. Comparison of plasma Aβ1-42 concentrations in patients with clinical diagnosis supported by corresponding CSF NDD results. Horizontal bars represent averages.

7 Fig. 2B. Comparison of plasma Aβ1-40 concentrations in patients with clinical diagnosis supported by corresponding CSF NDD results. Horizontal bars represent averages.

9 Fig. 2C. Comparison of plasma Aβ1-42/1-40 concentration ratios in patients with clinical diagnosis supported by corresponding CSF NDD results. Horizontal bars represent averages.
1

References


Table 1: Demographical data.

<table>
<thead>
<tr>
<th>Categorization</th>
<th>Groups</th>
<th>N</th>
<th>Age (SD)</th>
<th>gender (M+F)</th>
<th>positive/total genotyped</th>
<th>MMSE (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clinical &amp; Neuropsychological</td>
<td>D-AD</td>
<td>127</td>
<td>70.8 (7.7)</td>
<td>50+77</td>
<td>79/112 (71%)</td>
<td>22.8 (3.0)</td>
</tr>
<tr>
<td></td>
<td>D-O</td>
<td>25</td>
<td>66.6 (9.5)</td>
<td>14+11</td>
<td>10/21 (48%)</td>
<td>22.9 (3.2)</td>
</tr>
<tr>
<td></td>
<td>MCI-AD</td>
<td>137</td>
<td>67.4 (8.1)</td>
<td>73+64</td>
<td>59/115 (51%)</td>
<td>26.8 (2.2)</td>
</tr>
<tr>
<td></td>
<td>MCI-O</td>
<td>62</td>
<td>63.3 (8.9)</td>
<td>42+20</td>
<td>13/55 (24%)</td>
<td>27.2 (2.8)</td>
</tr>
<tr>
<td>Neurochemical (CSF-NDD)</td>
<td>NDD+</td>
<td>216</td>
<td>69.9 (7.7)</td>
<td>95+121</td>
<td>131/188 (70%)</td>
<td>24.3 (3.3)</td>
</tr>
<tr>
<td></td>
<td>NDD-</td>
<td>135</td>
<td>64.6 (8.9)</td>
<td>84+51</td>
<td>30/115 (26%)</td>
<td>26.6 (3.0)</td>
</tr>
<tr>
<td>Clinical, Neuropsychological &amp; Neurochemical</td>
<td>AD/NDD+a)</td>
<td>193</td>
<td>70.2 (7.6)</td>
<td>84+109</td>
<td>120/168 (71%)</td>
<td>24.2 (3.2)</td>
</tr>
<tr>
<td></td>
<td>O/NDD-b)</td>
<td>64</td>
<td>63.2 (9.2)</td>
<td>45+19</td>
<td>12/56 (21%)</td>
<td>26.6 (3.2)</td>
</tr>
</tbody>
</table>

1a) A combined group of subjects with early AD and corresponding MCI-AD, with neuropsychologic diagnosis confirmed by abnormal CSF biomarkers;

1b) A combined group of subjects with early other dementias (D-O) and corresponding MCI-O, with neuropsychologic diagnosis confirmed by normal CSF biomarkers;

3a) A combined group of subjects with early AD and corresponding MCI-AD, with neuropsychologic diagnosis confirmed by abnormal CSF biomarkers;

4a) A combined group of subjects with early other dementias (D-O) and corresponding MCI-O, with neuropsychologic diagnosis confirmed by normal CSF biomarkers;
Lewczuk et al. Fig. 1
Lewczuk et al. Fig. 2A
Lewczuk et al., Fig. 2B
Lewczuk et al., Fig. 2C