Electrophoretic separation of amyloid β peptides in plasma

In this prospective study, for the first time we have separated and quantified amyloid β (Aβ) peptides in the plasma of patients with Alzheimer’s disease (AD, n = 8) and age- and environment-matched healthy controls (n = 9) with urea-based Aβ-sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)/immunoblot. In addition to the Aβ peptides 1–37/38/39/40/42, which we recently identified as regular constituents of human cerebrospinal fluid (CSF), we have observed a novel electrophoretic band migrating slightly cathodically to Aβ1–42. Since a standard peptide with the amino acid sequence Aβ2–40 migrates in the same position, we hypothesize that this plasma-specific band may correspond to Aβ2–40. The concentration of Aβ peptides in the plasma has been approximately 100-fold lower compared to the CSF. Interestingly, the concentration of the two shortest peptides and the longest one of these considered here (i.e., Aβ1–37/38/42) have increased significantly when the samples have been frozen at −80°C before immunoprecipitation, while the ‘middle-length’ peptides (i.e., Aβ1–39/40) have not been affected by this procedure. We have not observed significant differences of the Aβ peptides concentrations between AD and control subjects.

Our method can be used to investigate the significance of plasma Aβ peptides in neurodegenerative disorders, and to monitor the efficiency of drugs with β/γ-secretase inhibitory potency.

Keywords: Alzheimer’s disease / Amyloid β / Neurodegeneration / Plasma

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1 Introduction

Among the candidate biomarkers to support diagnose of Alzheimer’s disease (AD), cerebrospinal fluid (CSF) concentrations of amyloid β peptides (Aβ peptides) and Tau protein(s) have been proven to fulfill the criteria for good AD diagnostic tests, as recently summarized by an expert review [1]. This is not surprising since these factors are directly involved in the pathologic events of the disease, namely deposition of senile plaques and formation of neurofibrillary tangles. CSF concentration of Aβ peptide ending at amino acid position 42 (Aβ42) was reported to be decreased, and Tau protein was reported increased in AD [2–9]. Moreover, recently published evidence shows that a combined analysis of biomarkers (Aβ42, total Tau protein, and Tau phosphorylated at the amino acid position 181, pTau181) discriminated correctly a subgroup of patients with mild cognitive impairment, who eventually developed AD, from those who did not [10].

Lumbar puncture is a relatively safe and uncomplicated procedure, and only a small ratio of AD patients complains about post-puncture complications [6, 11]. However, repunctures and follow-up measurements of CSF parameters are generally considered as inconvenient for patients, and thus there is a need to search for alternative body fluids as a possible source of relevant biomarkers. There are several hypothesis-driven rationales to speculate that blood and blood-derived fluids (serum and plasma) would fulfill criteria of such a source: (i) since the CSF stays in the direct

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Abbreviations: Aβ, amyloid β; AD, Alzheimer’s disease; ApoE, apolipoprotein E; CSF, cerebrospinal fluid; MMSE, mini-mental state examination; pTau181, Tau protein phosphorylated at the amino acid position 181

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contact with blood on the way of its flow in the spinal canal, several brain-derived factors have been found present also in the blood [12]; (ii) according to the concept of Felgenhauer and Beuche [13], brain-derived factors released in the regions of brain distant from the ventricles may predominantly leave the brain parenchyma through the blood-brain barrier into the blood instead of through the brain-CSF barrier into the CSF; (iii) parenteral administration of Aβ-specific monoclonal antibody facilitates a rapid efflux of brain-derived Aβ40 and Aβ42 into the plasma in the transgenic mice model expressing human mutated amyloid precursor protein gene [14, 15]; (iv) Aβ seems to be eliminated from the human brain parenchyma primarily into leptomeningeal arteries [16]. Moreover, carrier cells seem to facilitate the transport of Aβ peptides at the blood-brain barrier [17, 18]. Therefore, in this prospective study for the first time we have applied the urea version of the Bicine/bis-Tris/Tris/sulfate SDS-PAGE [2, 19, 20] to separate and quantify Aβ peptides in plasma of carefully selected patients with AD and non demented controls.

2 Materials and methods

2.1 Patients

The study was approved by the Ethics Committee of the University of Erlangen-Nürnberg. All patients gave their informed consent. For the purpose of this study, patients registered in the outward clinic of the Department of Psychiatry and Psychotherapy, University of Erlangen-Nürnberg were invited together with their relatives and/ or caregivers serving as control subjects. The group of Alzheimer’s disease (AD, n = 8) consisted of patients diagnosed according to the criteria of ICD-10, the National Institute of Neurological and Communicative Disorders, and the Stroke-Alzheimer’s Disease and Related Disorders Association (NINCDS-ADRDA) [21]. The group comprised three men and five women with a median age of 67.5 years (range, 50–79 years). The degree of mental impairment was assessed in all patients with the mini-mental state examination (MMSE) [22] showing the median score of 21.5 (range 18–23). The control group (CON, n = 9) comprised healthy subjects (eight men and one woman) without memory complaints. MMSE was performed in all persons, and revealed scores of 29–30. In the majority of cases, the subjects were relatives and caregivers of the AD patients living in the same environment. The median age of the control subjects was 63 years (range 47–81 years), and it did not differ significantly from the age of the AD patients.

2.2 Blood collection, sample treatment, and apolipoprotein E genotyping

Blood was collected from all subjects by venipuncture into a standard monovette containing EDTA (Sarstedt, Germany). Immediately after collection, the blood was centrifuged (1600 × g, 10 min, +4°C), and the plasma was divided into two aliquots, 500 µL each: one of them was immediately frozen at −80°C, and the other one was immunoprecipitated immediately before freezing. Apolipoprotein E (ApoE) genotyping was performed with Inno-LiPA ApoE kits (Innogenetics, Ghent, Belgium) on genomic DNA purified from blood with QiAamp DNA Mini Kit (Qiagen, Hilden, Germany), using a thermocycler from Biometra (Goettingen, Germany) according to a previously described protocol [23]. The results were statistically analyzed with regard to the presence or absence of the ε4 allele of the ApoE gene.

2.3 Immunoprecipitation of Aβ peptides and their electrophoretic separation

For immunoprecipitation, magnetic sheep anti-mouse immunoglobulin G (IgG) Dynabeads M-280 (Dynal, Hamburg, Germany) were incubated overnight at +4°C with monoclonal Aβ amino terminal-selective antibody, 1E8 (nanoTools, Teningen, Germany) according to the manufacturer’s protocol. Five hundred µL of either fresh or frozen/thawed plasma was added to 200 µL fivefold concentrated RIPA detergent buffer [2, 25 µL preactivated magnetic Dynabeads (1 µL mAb 1E8/1.68 × 10⁷ beads), and 300 µL H₂O. Samples were then incubated overnight at +4°C under rotation, following washing of the beads four times with PBS/0.1% bovine serum albumin, and once with 10 mM Tris/HCl, pH 7.4. For Aβ–SDS-PAGE/immunoblot, bound Aβ peptides were eluted by heating the samples to 95°C for 5 min with 25 µL sample buffer I [2]. For separation of Aβ peptides, the urea version of the Bicine/bis-Tris/sulfate SDS-PAGE was used as described by Wittfang et al. [2, 19]. Briefly, immunoprecipitated plasma (5 µL immunoprecipitate) in sample buffer I was applied to a gel slot. To allow quantification of Aβ peptides, five dilutions of the standard Aβ peptide mixture composed of synthetic Aβ peptides (Aβ1–38, Aβ1–40, and Aβ1–42 were from Bachem, Bubendorf, Switzerland, and other peptides were synthesized as described previously [24]) were applied on the gels along with the samples. Gels were run at room temperature for 1 h at a constant current of 24 mA/gel using the MiniProtean III electrophoresis unit (Bio-Rad, Munich, Germany). Next, semidy Western blotting was performed using PVDF membranes, which were then immunostained overnight with monoclonal amino terminal-selective antibody, 1E8 (nanoTools) at +4°C. After the
winding step, the membranes were incubated for 1 h at room temperature with an anti-mouse biotinylated antibody (Vector Laboratories, Burlingame, CA, USA), washed, and horseradish peroxidase-coupled streptavidin (Amersham Biosciences, Freiburg, Germany) was added for 1 h. After the final washing step, chemiluminescence was visualized with ECLPlus solution (Amersham Pharmacia) according to the protocol of the manufacturer, using the CCD camera system (FluorSMax Multilamger, Bio-Rad). To evaluate analytical specificity of the method as well as possible interactions of Aβ peptides with proteins, two control experiments were performed with human CSF. In the first, the complete method was performed with and without 1E8 coupled to immunoprecipitating magnetic beads, and the separated proteins were silver-stained according to the protocol described elsewhere [19]. In the second control experiment, Aβ-specific antibodies were eliminated from the experimental protocol, either from the immunoprecipitating step (incubation of CSF with nonactivated magnetic beads) or from the immunostaining step.

2.4 Coefficients of variations of Aβ peptides’ measurements

To assess the variation of the method, one sample (pooled plasma from four donors, 500 μL each) was immunoprecipitated and run n = 16 times on two gels together with appropriate standards. Coefficients of variations (CVs) were calculated for all five peptides (Aβ1–37/38/39/40/42).

2.5 Statistical analysis

The results are expressed as medians and ranges. Statistical comparison between groups was performed with Mann-Whitney’s U-test (Statistica 6.0, Statsoft, USA). The comparison of the results obtained before versus after freezing of plasma was performed with Wilcoxon’s test. The correlations between parameters were assessed with Spearman’s correlation rank. A p-level less than 0.05 was considered significant.

3 Results

3.1 Separation of Aβ peptides in human plasma with electrophoresis: evidence of a novel Aβ peptide present in plasma but not in CSF

Representative patterns of plasma and, for comparison, CSF Aβ peptides separated with the urea version of the Bicine/bis-Tris/Tris/sulfate Aβ-SDS-PAGE/immunoblot are presented in Fig. 1a. According to the Aβ standards applied (lane 1), five peptides are present in the plasma (lane 3) similarly to the CSF (lane 4), namely Aβ1–37/38/39/40/42. Surprisingly, in addition to this ‘Aβ peptide quintet’ we observed in plasma also a band migrating slightly cathodically to Aβ1–42 (line 3). Since synthetically manufactured Aβ peptide with the amino acid sequence of 2–40 (Aβ2–40) comigrates correspondingly (lane 2), we hypothesize that the novel Aβ peptide might be Aβ2–40.

Figure 1. (a) Representative electrophoretic separation of Aβ peptides in human plasma. Lane 1, mixture of the standard synthetic Aβ peptides: 1–37/38/39/40/42 and 2–42; lane 2, synthetic Aβ2–40; lane 3, representative plasma sample; lane 4, representative cerebrospinal fluid sample. (b) Silver staining of the complete method with (lane 1) and without (lane 2) coupling of the immunoprecipitating antibody (1E8) to the beads. Similar patterns of both separations suggest presence of unspecific binding of CSF proteins to the beads. (c) Negative controls of the method’s specificity. Lane 1, standard peptides’ mixture (same as in (a), lane 1); lane 2, CSF proteins immunoprecipitated with 1E8, and immunostained with the detection antibody (1E8); lane 3, complete method after exclusion of 1E8 from the immunoprecipitating step; lane 4, complete method after exclusion of immunoprecipitating and immunostaining antibodies; lane 5, exclusion of the detection antibody. Note that unspecific reaction of the CSF proteins to the beads (lane 3) and unspecific reaction during the immunostaining step (lane 5) occur exclusively in the higher molecular range of the method, and do not affect the region of Aβ peptides.
3.2 Evaluation of the specificity of the method

The results of the silver staining of Aβ peptides in human CSF precipitated with 1E8 coupled to the magnetic beads or by unspecific reaction with nonactivated beads are presented in Fig. 1b. There is no obvious difference between the pattern of proteins immunoprecipitated by Aβ-specific antibody (lane 1), and unspecific binding of the proteins to the plain beads (lane 2). The separation patterns after exclusion of the immunoaffinity steps from the experimental procedure are presented in Fig. 1c. Lane 1 shows the results of the separation of the standard Aβ peptides mixture, lane 2 presents the whole method including Aβ-specific immunoprecipitation with activated beads and Aβ-specific immunostaining. In lane 3, the separation is presented with Aβ-specific antibody excluded from the immunoprecipitation step. Lane 4 presents the pattern after exclusion of both immunoaffinity steps (i.e., with neither activation of the beads, nor immunostaining). Lane 5 shows the separation pattern after Aβ-specific immunoprecipitation followed by exclusion of immunostaining. Comparison of the antibody elimination modifications of the protocol reveals unspecific binding during immunoprecipitation and immunostaining (lanes 3 and 5, respectively), however, these reactions are present exclusively in the higher-molecular-mass region, and do not afflict the region where Aβ peptides are present.

3.3 Immunoprecipitation before versus after freezing: absolute values, percentages, and quotients

The effects of the samples freezing prior to the immunoprecipitation on the Aβ peptides concentrations are presented in Fig. 2. Freezing of the plasma samples resulted in increase of Aβ1–37 concentration (Fig. 2a) from 36.6 pg/mL (range, 17.4–59.1) to 38.6 pg/mL (24.2–59.1, p < 0.05).

![Figure 2](image-url)

Figure 2. Influence of preanalytical sample handling on the Aβ peptides' concentration. Each spot represents results from a single subject (black triangles, AD; open diamonds, controls). The concentration measured before freezing of the sample is given as horizontal coordinate and the concentration measured after freezing of the sample at −80°C is given as vertical coordinate of each spot. Spots situated above the marked 45° line represent the cases with increased concentration of a given Aβ peptide species after freezing of the sample compared to the result obtained before the freezing. Results of (a) Aβ1–37; (b) Aβ1–38; (c) Aβ1–39; (d) Aβ1–40; (e) Aβ1–42; (f) total Aβ peptides.

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The concentration of Aβ1–38 (Fig. 2b) increased after freezing of the samples from 36.9 pg/mL (25.5–55.3) to 40.8 pg/mL (26.4–58.1; p < 0.05). Interestingly, freezing of the samples prior to immunoprecipitation did not influence the results of Aβ1–39 (Fig. 2c) as the concentration of this biomarker only slightly increased from 31.1 pg/mL (18.2–47.5) to 32.9 pg/mL (23.9–47.3; not significant). Similarly, a significant effect of the initial plasma freezing on the Aβ1–40 concentration (Fig. 2d) was not observed, as its concentration increased only slightly from 145.7 pg/mL (75.6–232.6) to 159.4 pg/mL (102.9–224.9; not significant). The concentration of Aβ1–42 (Fig. 2e) significantly increased after the initial freezing of the samples from 37.1 pg/mL (17.8–57.0) to 40.4 pg/mL (22.8–57.3; p < 0.05). The total Aβ peptides concentration (Fig. 2f) turned out to be highly significantly influenced by the initial freezing process as it increased from 288.9 pg/mL (194.1–448.5) to 311.6 pg/mL (217.9–446.1; p < 0.01).

Neither the ratio of a particular Aβ species to the total concentration of Aβ peptides (Aβ peptide percentage ratio) nor the Aβ peptides concentration quotient (i.e., Aβ1–42/Aβ1–40) were significantly influenced by the freezing of the plasma prior to the immunoprecipitation (Fig. 3).

### 3.4 Aβ peptides in AD versus controls: absolute values, percentages, and quotients

We did not observe any significant differences between AD and control subjects with regard to any biomarker measured in this study (data not shown).

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**Figure 3.** Influence of preanalytical sample handling on the ratios of Aβ peptides in plasma. Each spot represents results from a single subject (black triangles, AD; open diamonds, controls). The ratios of the peptides measured before freezing of the sample are given as horizontal coordinate and the ratios measured after freezing of the sample at −80°C are given as vertical coordinate of each spot. Spots situated above the marked 45° line represent the cases with increased ratio of a given Aβ peptide species after freezing of the sample compared to the result obtained before the freezing. Percentage ratio of the concentrations of (a) Aβ1–37 to total Aβ peptides; (b) Aβ1–38 to total Aβ peptides; (c) Aβ1–39 to total Aβ peptides; (d) Aβ1–40 to total Aβ peptides; (e) Aβ1–42 to total Aβ peptides; (f) Aβ1–42/Aβ1–40.
3.5 Correlation of Aβ peptides (absolute values, percentages, and quotients) with age, sex, MMSE, and ApoE genotype

A correlation of plasma Aβ peptides with the age of the patients was not observed in our study (data not shown). Similarly, none of the factors correlated significantly with the gender of the patients. None of the biomarkers studied correlated significantly with the MMSE score. Interestingly, the subjects with the ApoE ε4 allele had a significantly higher percentage of Aβ1–38 when immunoprecipitation was performed on fresh samples compared to the subjects without this allele (medians, 13.5% and 12.4%, respectively, p < 0.05).

3.6 Intra-assay coefficients of variations for plasma Aβ peptides

Coefficients of variations of the Aβ peptides’ concentrations turned out to be in the range of 14.0–18.5%. Similarly, CVs of the Aβ peptides’ quotients were in the range of 11.5–18.5%.

4 Discussion

In this prospective study, we present the results of electrophoretic separation of Aβ peptides in human plasma. Similarly to the results of the CSF separation as reported by our group before [2, 4], ‘Aβ peptide quintet’ of Aβ1–37/38/39/40/42 is present also in the plasma, however, in addition to these peptides, a band migrating at the position of synthetic Aβ2–40 was revealed. We did not observe any differences in the concentration of plasma Aβ peptides between AD and controls, however, amino acid sequence-specific alterations were observed in the Aβ peptides’ concentrations in the samples immunoprecipitated immediately after blood collection compared to those frozen before immunoprecipitation.

With the urea version of the Bicine/bis-Tris/Tris/sulfate Aβ-SDS-PAGE/immunoblot, we were recently able to separate Aβ peptides from human CSF and post mortem brain tissue, as well as cell culture supernatants [2, 4, 25]. The results reported in the present study extend the application of this method to separation of Aβ peptides in human plasma. Similarly to the results obtained in CSF, Aβ1–40 is the most prominent Aβ peptide in the plasma, and the overall concentration of Aβ peptides in the plasma is approximately 100-fold lower compared to the concentration in CSF [2, 4]. A similar gradient of Aβ peptides’ concentration between plasma and CSF was reported also in mice [26], however, similarly to virtually all methods based on comparisons of the signal’s intensity in a standard and unknown sample, it is difficult to make conclusions on the absolute abundance of individual peptides. The CSF/plasma gradient might be explained by the fact that Aβ peptides are predominantly released from the brain tissue, and thus, similarly to all brain-derived proteins/peptides reported so far (for review, see [12]), their concentration on ‘the CSF side’ of the blood-CSF barrier is much higher than on the other side. However, some evidences exist that Aβ peptides may be released from other tissue in addition to the brain [27, 28], and therefore the low Aβ concentrations observed in the plasma may result from the binding to carrier proteins or conformational changes masking the epitopes.

Compared to the Aβ peptides pattern in the CSF, Aβ2–42 was not present in the plasma, however, we observed a slight but distinct band migrating cathodically to Aβ1–42. This band may represent Aβ1–40, since the synthetic standard peptide of this sequence migrated in this same position, however, also other sequences as well as other post-translational modifications cannot be excluded, and the further studies are now being performed to reveal the amino acid sequence of this molecule. Aβ peptide with the amino acid sequence of 2–40 was reported in the brains of AD patients by Huse et al. [29]. Interestingly, the presence of peptides characteristic for the plasma, i.e., not present in the CSF, was reported by the group of Beyreuther [30] in the original paper describing G2–10 and G2–11 monoclonal antibodies. However, since the system used then separated Aβ1–40 and 1–42 but neither Aβ1–37/38 nor 39, it is difficult to speculate whether the plasma-characteristic band observed by this group was the same we observed in our system or they are two independent, plasma-specific Aβ peptides. Albeit the precise sequence of this novel peptide is now under further studies, our data clearly support the hypothesis of Ida et al. [30] that the presence of the plasma-specific peptide(s) points at the release of at least some of Aβ peptides also from the tissue(s) other than the central nervous system.

As an interesting finding, we observed significant and sequence-specific alterations of the concentrations resulting from the modifications of the sample handling. The concentration of the two shortest peptides and the longest one of these reported here (i.e., Aβ1–37/38/42) increased significantly when the samples were frozen at −80°C before immunoprecipitation, while the ‘middle-length’ peptides (i.e., Aβ1–39/40) were not affected by this procedure. We suggest that this observation should be considered as an important aspect of the preanalytical sample handling. Since neither the ratios of a given Aβ peptide species to the total amount of Aβ peptides nor Aβ peptide concentration quotient (like, e.g., Aβ42/40) are affected by this type of preanalytical handling, we assume...
that Aβ quotients represent a more reliable approach to the evaluation of the measured data than the ‘rough’ concentrations.

In our study, antibodies applied to immunoprecipitate and immunostain Aβ peptides were shown to react specifically with the peptides in question. Unspecific interactions of the antibodies were observed in the high-molecular mass region of the gels but not in the region of Aβ peptides. Silver staining revealed the presence of several electrophoretic bands, however, since they were present also after exclusion of immunoprecipitation (precipitation of the CSF with plain beads), we assume that they result from unspecific interactions between CSF proteins and CSF peptides in question. Unspecific interactions of Aβ-binding factors in human body fluids, as such interactions of Aβ peptides and its precursor protein are known and extremely important under (patho)physiological conditions [31, 32].

The data published so far are conflicting with regard to disease-specific alterations of Aβ peptide concentration in plasma. These discrepancies may result from different methods used, especially since the majority of them recognize only some of the Aβ peptides (e.g., Aβ40 and Aβ42). In addition, a considerable source of discrepancies between studies might also include non-standardized preanalytical sample-handling protocols, and different populations studied, e.g., patients and controls exposed to different environmental conditions. To our best knowledge, the present study is the first one performed with Aβ-SDS-PAGE/immunoblot, which enabled a more detailed separation of Aβ peptides in plasma compared, e.g., to ELISAs or a conventional SDS-PAGE/immunoblot [30], and we were able to identify and quantify not only Aβ40 and Aβ42, as reported in other studies, but also other species, for which currently ELISAs are not available. Moreover, since there are evidences that environmental factors can influence the development of AD disease by interfering with Aβ and Tau metabolism [33], we designed our study to reduce the possible influence of such factors, as the subjects of the control group (in the majority of cases relatives and/or caregivers of the AD patients) were exposed to similar conditions as the AD patients (food, water quality, possible air pollutions, etc.). In accordance with findings of the previous studies of Tamaoka et al. [34] and Ida et al. [30], in the present study we did not observe changes in the plasma Aβ peptide concentrations in AD compared to the control population. In accordance with these data, DeMattos et al. [15] did not observe any correlation between the baseline levels of plasma Aβ40 or Aβ42 and brain Aβ burden in PDAPP transgenic mice expressing human mutated APP gene. However, Mehta et al. [35] reported increased Aβ40 concentrations in the AD group while Aβ42 was unchanged, and Mayeux et al. [36] published increased baseline plasma Aβ40 and Aβ42 as predicting factors in the group of healthy individuals who developed AD approximately 3.6 years later. Interestingly, Bornebroek et al. [37] found decreased plasma Aβ42 concentration in the patients with Dutch mutation in the APP sequence [37].

In conclusion, for the first time we report the electrophoretic separation of the Aβ peptide species in human plasma. Compared to the results in CSF, the pattern of Aβ peptides in plasma characterizes with qualitative and quantitative differences. Interestingly, some Aβ peptides in plasma are more prone to preanalytical sample handling, like, e.g., freezing of the sample before immunoprecipitation, than the others, and hence, for the evaluation of the data, we propose analysis of Aβ peptide quotients, rather than the concentrations of the peptides themselves. The concentrations of Aβ peptides in plasma are not altered in AD, nevertheless, we believe that this report provides a methodology for further studies on plasma Aβ peptides in other relevant neurodegenerative disorders.

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5 References
