



A Specific Reduction in A β _{1–42} vs. a Universal Loss of A β Peptides in CSF Differentiates Alzheimer's Disease From Meningitis and Multiple Sclerosis

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OPEN ACCESS

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Received: 31 January 2018

Accepted: 04 May 2018

Published: 24 May 2018

Citation:

Spitzer P, Lang R, Oberstein TJ, Lewczuk P, Ermann N, Huttner HB, Masouris I, Kornhuber J, Ködel U and Maler JM (2018) A Specific Reduction in A β _{1–42} vs. a Universal Loss of A β Peptides in CSF Differentiates Alzheimer's Disease From Meningitis and Multiple Sclerosis
Front. Aging Neurosci. 10:152.
doi: 10.3389/fnagi.2018.00152

A reduced concentration of A β _{1–42} in CSF is one of the established biomarkers of Alzheimer's disease. Reduced CSF concentrations of A β _{1–42} have also been shown in multiple sclerosis, viral encephalitis and bacterial meningitis. As neuroinflammation is one of the neuropathological hallmarks of Alzheimer's disease, an infectious origin of the disease has been proposed. According to this hypothesis, amyloid pathology is a consequence of a microbial infection and the resulting immune defense. Accordingly, changes in CSF levels of amyloid- β peptides should be similar in AD and inflammatory brain diseases. A β _{1–42} and A β _{1–40} levels were measured in cerebrospinal fluid by ELISA and Western blotting in 34 patients with bacterial meningitis ($n = 9$), multiple sclerosis ($n = 5$) or Alzheimer's disease ($n = 9$) and in suitable controls ($n = 11$). Reduced concentrations of A β _{1–42} were detected in patients with bacterial meningitis, multiple sclerosis and Alzheimer's disease. However, due to a concurrent reduction in A β _{1–40} in multiple sclerosis and meningitis patients, the ratio of A β _{1–42}/A β _{1–40} was reduced only in the CSF of Alzheimer's disease patients. Urea-SDS-PAGE followed by Western blotting revealed that all A β peptide variants are reduced in bacterial meningitis, whereas in Alzheimer's disease, only A β _{1–42} is reduced. These results have two implications. First, they confirm the discriminatory diagnostic power of the A β _{1–42}/A β _{1–40} ratio. Second, the differential pattern of A β peptide reductions suggests that the amyloid pathology in meningitis and multiple sclerosis differs from that in AD and does not support the notion of AD as an infection-triggered immunopathology.

Keywords: meningitis, amyloid, Alzheimer's disease, multiple sclerosis, biomarker, dementia, neuroinflammation, neurodegeneration

INTRODUCTION

In addition to amyloid plaque deposition, neuroinflammation is one of the neuropathological hallmarks of Alzheimer's disease (AD) (Heneka et al., 2015). Viral, bacterial and even fungal antigens have been found in association with the pathognomonic amyloid-beta (A β) depositions (Itzhaki, 2014; Little et al., 2014; Piacentini et al., 2015; Pisa et al., 2015; Zhan et al., 2016). The secretion of A β peptides during inflammation and the observation of anti-infective properties of A β peptides support the idea that the production of A β peptides provides an immune defense (Spitzer et al., 2010, 2016; Condic et al., 2014; Kumar et al., 2016). These and other findings have culminated in the formulation of the infection hypothesis of AD (Miklosy, 2011). According to this hypothesis, amyloid deposition is the consequence of ongoing neuroinflammation evoked by a pathogen that evades the immune system.

In the diagnosis of AD, decreased levels of soluble A β peptide 1-42 (A β ₁₋₄₂) in the cerebrospinal fluid (CSF) are widely accepted as a surrogate for brain amyloidosis (McKhann et al., 2011; Blennow and Zetterberg, 2015; Jack et al., 2016; Lewczuk et al., 2017). Fitting perfectly into the infection hypothesis, reduced levels of A β ₄₂ are also found in patients with brain infections, such as bacterial meningitis, herpes encephalitis or human immunodeficiency virus (HIV)-associated dementia (Sjögren et al., 2001; Krut et al., 2013). A β ₁₋₄₂ is generated during cleavage of amyloid precursor protein (APP). In addition to A β ₄₂, several other A β peptide variants with different C- and N-termini are generated during this process (Wiltfang et al., 2002). It is hypothesized that it is not the overproduction of A β ₄₂ that leads to its deposition but rather an imbalance of the different A β peptide variants (Hasegawa et al., 1999; Jan et al., 2008). Consequently, the concentration of A β ₁₋₄₂ in relation to A β ₁₋₄₀, the most abundant A β peptide variant, was found to be superior to A β ₁₋₄₂ alone as a biomarker for AD (Lewczuk et al., 2004, 2015a,b; Janelidze et al., 2016; Niemantsverdriet et al., 2017). If this imbalance between A β ₄₂ and A β ₄₀ that is observed in AD could also be found in inflammatory brain diseases, those data would further support the infection hypothesis of AD.

Therefore, this study investigates whether the changes in A β ₁₋₄₂ and A β ₁₋₄₀ levels in CSF during AD resemble those observed in multiple sclerosis (MS) and bacterial meningitis and whether the A β -ratio (A β ₁₋₄₂/A β ₁₋₄₀) differs between these diseases.

MATERIALS AND METHODS

Patients

Samples were collected in the memory clinic of the Department of Psychiatry, Erlangen, the Neurological Department, Erlangen, and the Neurological Department of Ludwig-Maximilian-University (LMU), Munich. Patients or their legal representatives provided their informed consent, and the study protocol was approved by the ethics committee of the University Hospital Erlangen-Nuremberg (project no. 3987) and of LMU Munich (project no. 349-15 and 174-11). Patients included in this study underwent psychiatric, neurological, medical and routine

laboratory examinations. Additionally, an MRI scan and a CSF analysis were performed. The CSF of patients with meningitis was drawn under emergency conditions, so oligoclonal bands were not routinely determined. The diagnosis of AD was made according to the National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA) criteria, taking into account the A β ₄₂/A β ₄₀ ratio and the total tau and phospho-tau levels in the CSF (Albert et al., 2011; McKhann et al., 2011). The Erlangen Score (ES) algorithm (Lewczuk et al., 2009, 2015a) was also used to classify patients: all AD patients had an ES \geq 3, and all controls had an ES \leq 1. In reference to the criteria suggested by Jack et al., the patients within the AD group were classified according to the presence (+) or absence (-) of A β (A), neurofibrillary tangles (T) and neurodegeneration (N) as A+/T+/N+, whereas those in the control group were A-/T-/N- (Jack et al., 2016). Amyloid pathology was evaluated by the A β ₄₂/A β ₄₀ ratio, tau pathology was evaluated by phospho-tau levels, and neurodegeneration was evaluated by total-tau levels and temporo-parietal atrophy in the MRI scan. Patients with intermediate signs of AD pathophysiology were excluded. MS was diagnosed according to the revised McDonald criteria (Polman et al., 2011), and meningitis was diagnosed according to the European Society of Clinical Microbiology and Infectious Diseases (ESCMID) guidelines (van de Beek et al., 2016). The control group comprised five patients with tension headache, one with schizophrenia, two with idiopathic epilepsy and one with a depressive episode. CSF samples were collected in polypropylene tubes, centrifuged within 24 h after sampling and stored in aliquots at -80°C until further use.

ELISA

A β ₁₋₄₀ and A β ₁₋₄₂ levels were quantified with certified ELISA tests (IBL international GmbH, Hamburg, Germany) according to the manufacturer's instructions. Samples were thawed immediately before the analysis and diluted 1:20 in reagent diluent. Then, 100 μl of each sample, standard, positive control or blank were added in duplicate to a pre-coated microtiter plate. After incubation for 2 h, the plate was washed, and horseradish peroxidase (HRP)-conjugated detection antibody (clone 82E1) was added for another 1 h. After washing, the chromogenic substrate 3,3',5,5'-tetramethylbenzidine (TMB) was added, and the plate was read. The analyses were performed under careful quality control, and a measurement was regarded as valid if the range-to-average coefficient of the duplicate measurements was below 20%.

SDS-PAGE/Western Blotting

The immunoprecipitation, urea-sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting procedures have been described in detail before (Wiltfang et al., 2002; Oberstein et al., 2015). In short, immunoprecipitation was carried out with mouse anti-amyloid antibody 6E10 (Biolegend, formerly Covance, Koblenz, Germany), reactive to amino acids 1-16 of the A β peptide, covalently bound to magnetic sheep anti-mouse Dynabeads[®] M-280 (10 mg/ml; Dynal, Hamburg, Germany). Samples

were loaded onto bicine-SDS gels containing 8 M urea and separated for 55 min at 25 mA/gel. After semi-dry blotting onto polyvinylidene fluoride (PVDF)-membranes with a discontinuous buffer system, immunolabeling was performed with the anti A β_{1-x} antibody 82E1, which only recognized A β peptides starting with amino acid 1 (1:1,000 in phosphate-buffered saline-Tris (PBS-T); IBL, Hamburg Germany). ECL[®] (enhanced chemiluminescence) prime (GE Healthcare, Freiburg, Germany) was used to develop the immunoblots, and chemiluminescence was detected using an Amersham[®] Imager 600 instrument (GE Healthcare, Freiburg, Germany). The antibodies (6E10 for the immunoprecipitation and 82E1 for the detection) were chosen to achieve the highest possible sensitivity in this system.

Statistical Analysis

Statistical analysis was carried out with Prism[®] 6.0 (GraphPad Software Inc., La Jolla, CA, USA). Assuming a Gaussian distribution, parametric ANOVAs for independent samples followed by Fisher's *post hoc* tests were used to compare measurements among the different groups. To evaluate the diagnostic accuracy of the A β_{1-42} /A β_{1-40} ratio, a receiver operating characteristic (ROC) curve was generated. Pearson's correlation coefficient (*r*) was calculated to investigate the associations between different analytes. The results are presented as the mean with standard deviations and were considered to be significant at a *p*-value < 0.05.

RESULTS

Study Population

Samples were collected according to the same standard operating procedures at the Department of Psychiatry, Erlangen, and the Neurology Departments in Erlangen and Munich. Due to the ages at which the different pathologies typically occur, the samples could not be matched for age. However, there was no statistically significant age difference between the patients with meningitis and those with AD or between the controls and the patients with MS. The patients with meningitis had increased peripheral leucocyte counts, increased levels of C-reactive protein (CRP) and CSF protein, cells and erythrocytes and a higher CSF/serum albumin ratio. Patients with MS had slightly increased CSF leucocyte counts and positive oligoclonal bands in the CSF (Table 1).

Reduced A β_{1-42} in AD, MS and Meningitis

All measurements were performed in the same laboratory to avoid inter-center variations. A β_{1-40} and A β_{1-42} levels were quantified by ELISA. One patient in the meningitis group had an A β_{1-42} concentration below the lower limit of quantification and was excluded from further analysis. As expected, a reduced concentration of A β_{1-42} was found in AD samples. Additionally, in MS and bacterial meningitis, concentrations of A β_{1-42} were reduced (Figure 1A). The concentration of A β_{1-40} also tended to be reduced in MS and meningitis but not in AD (Figure 1B). As a consequence, the ratio of A β_{1-42} /A β_{1-40} was reduced only in AD (Figure 1C). No significant correlations between the increased

TABLE 1 | Patient characteristics—Patient characteristics are presented as the mean (standard deviation).

	Con	MS	Meningitis	AD
N (female)	9 (3)	5 (4)	9 (5)	11 (6)
Age [years]	44.1 (19.7)	36.4 (4.6)	64.8 (19.3)	69.9 (11.2)
Blood leukocytes [μ l]	8.6 (4.1)	6.9 (1.6)	16.9 (7.1)	6.4 (1.7)
Serum CRP [mg/l]	6.6 (13.4)	1.8 (1.3)	227.3 (100.6)	2.2 (2.4)
OCB (+/−)	0/9	5/0	1/2	0/11
Total protein (CSF) [mg/l]	583.3 (113.9)	464.0 (166.1)	4500 (3430)	370.4 (109.7)
Albumin ratio	6.0 (2.2)	7.3 (2.8)	85.0 (70.0)	6.4 (2.1)
Leukocytes (CSF) [μ l]	1.4 (1.3)	22.6 (14.9)	9543 (9827)	1.4 (0.9)
Erythrocytes (CSF) [μ l]	5.0 (10.3)	1.5 (1.3)	1295 (2015)	11.2 (22.7)

N, number; CON, control; MS, multiple sclerosis; AD, Alzheimer's disease; CRP, C-reactive protein; OCB, oligoclonal IgG bands on isoelectric focusing; CSF, cerebrospinal fluid.

values of erythrocytes, leukocytes or protein and the reduced concentrations of CSF A β_{1-40} or A β_{1-42} were found in patients with meningitis. The established threshold of 0.05 allowed for the differentiation of AD patient samples from control samples and samples from MS and meningitis patients with 100% sensitivity and 91% specificity.

Reduced Total Amyloid in MS and Meningitis but Not AD

CSF from representative patients with bacterial meningitis, multiple sclerosis and AD as well as one control was loaded directly onto SDS gels containing 8 M urea (Figure 2A). To control for artificially low concentrations of A β peptides due to the increased protein concentrations in meningitis, an additional separation of CSF from two patients with meningitis and two controls after immunoprecipitation with the 6E10 antibody was carried out (Figure 2B). The detection was performed with the anti-amyloid 82E1 antibody, which is specific for A β_{1-x} variants.

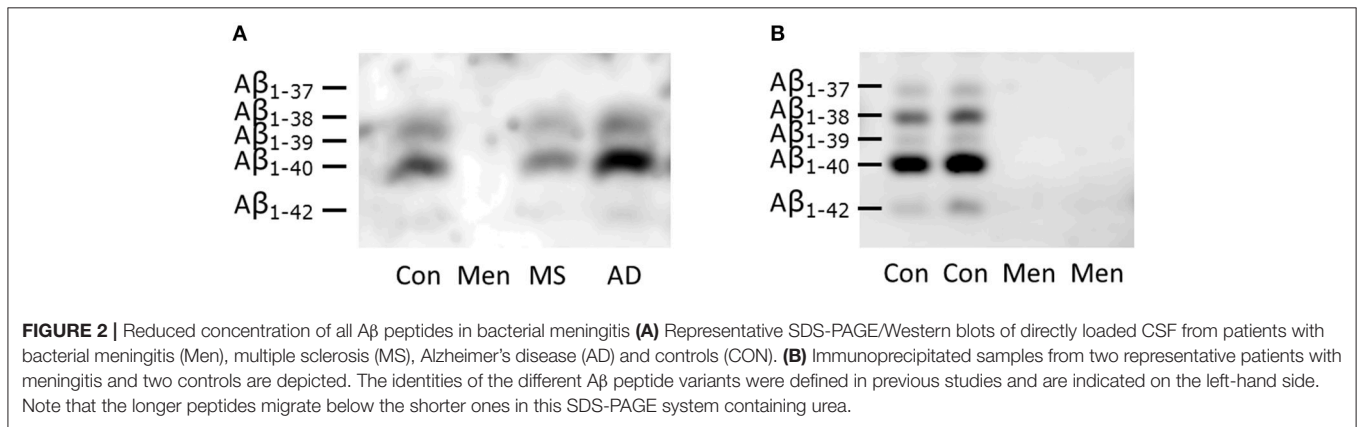
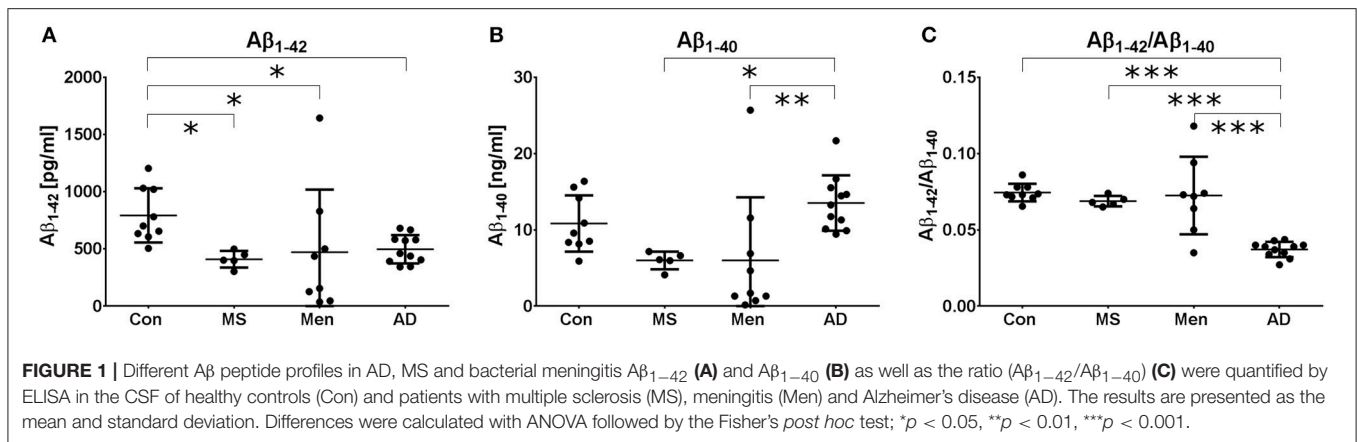
Reduced concentrations of all A β peptide variants were observed in the Western blots of CSF from meningitis patients (Figure 2). The concentration even dropped below the lower limit of detection.

DISCUSSION

The concentration of A β_{1-42} in CSF is reduced in patients with AD, MS and bacterial meningitis. However, the ratio of A β_{1-42} /A β_{1-40} is reduced only in AD, while the concentrations of all A β peptide variants are similarly reduced in the CSF of patients with bacterial meningitis.

This is the first study to show the distinct changes in A β peptide variants in the CSF during AD compared to inflammatory brain diseases.

Nevertheless, the study has several limitations. The sample size is relatively small, and an exact matching of age and sex was not possible. Sample matching is nearly impossible for age because AD is usually diagnosed above the age of 60, while patients with MS become symptomatic before the age of 50. However, the CSF concentrations of A β



peptides have been reported to be relatively stable throughout life and to be independent of sex in healthy subjects (Resnick et al., 2015).

To reduce the possible center effects, a balanced composition of the control and the meningitis groups was achieved. However, the samples for the MS group were all collected in Munich, while the samples for the AD group were all collected in Erlangen. However, the preanalytical handling of the samples was identical at all sites, and measurement of the A β peptides by ELISA was carried out in a single laboratory, as was recommended to effectively reduce center effects (Mattsson et al., 2013).

While A β_{1-42} is reduced in all three diseases, the observation that the A β ratio is reduced in AD but not in meningitis or MS points to a better diagnostic performance of the A β ratio. This improved performance has also been previously shown for the differential diagnosis of AD vs. non-neurodegenerative neuropsychiatric diseases, Lewy-body dementia, Parkinson's disease dementia or vascular dementia (Lewczuk et al., 2015b; Janelidze et al., 2016). Several others have therefore suggested to replace the measurement of A β_{1-42} with the A β ratio for AD diagnosis (Blennow and Zetterberg, 2015). Our study suggests that the A β ratio not only is able to increase the diagnostic performance in the differential diagnosis of neurodegenerative diseases but also helps to differentiate AD from neuroinflammatory diseases.

In the context of the infection hypothesis of AD, the observed differences among AD, MS and meningitis point to different pathophysiological processes behind the changes in CSF A β_{1-42} concentrations. While the processing of APP in AD is changed in a way that leads to an imbalance between the different A β peptide variants and allows the accumulation of A β_{1-42} into amyloid plaques, it was hypothesized that APP processing is impeded during CNS infections (Krut et al., 2013). Reduced concentrations of secreted (s)APP α and sAPP β during meningitis support this interpretation (Krut et al., 2013). An alternative explanation is that serum proteins entering the CSF after breakdown of the blood brain barrier in meningitis might mask the detection of A β peptides. However, others have found no differences in A β peptide concentrations via ELISA after adding proteins to the CSF (Bjerke et al., 2010), and our data show no correlation between increased CSF protein concentrations and reduced concentrations of A β peptides. Additionally, we performed immunoprecipitation to release the A β peptides from their protein bonds. We suggest that the amyloid peptides build complexes around the invading pathogens and are therefore no longer measurable in the CSF (Spitzer et al., 2016). By agglutinating microorganisms and exerting a direct antimicrobial activity, A β peptides have been shown to accumulate around microorganisms *in vitro* (Soscia et al., 2010; Torrent et al., 2012; Kumar et al., 2016; Spitzer et al., 2016). The increased

expression and distinct processing of APP during immunological activation *in vitro* also favors the idea of an immunological function and an increased consumption of A β peptides during infections and neuroinflammation in MS (Maler et al., 2008; Sondag and Combs, 2010; Spitzer et al., 2010). Further research is necessary to analyze the composition and fate of the A β peptides accumulating around the invading pathogens.

Current knowledge of inflammation-induced changes in A β peptide metabolism is mainly based on studies of acute infections (Spitzer et al., 2010; Kumar et al., 2016). Although MS is not an infectious disease, we used samples from MS patients to investigate the A β peptide levels in a chronic inflammatory disease. Mattsson and his colleagues measured the levels of A β_{38} , A β_{40} , and A β_{42} in patients with chronic neuroborreliosis (Mattsson et al., 2010). In both studies investigating two different entities causing chronic neuroinflammation, the concentrations of all measured A β peptide variants were reduced (Mattsson et al., 2010). Therefore, it seems that chronic neuroinfection and neuroinflammation are also associated with reduced levels of all A β peptides.

Taken together, the results of this study point to different mechanisms resulting in reduced A β_{1-42} levels in the CSF of patients with AD, bacterial meningitis or MS. The A β_{1-42} /A β_{1-40} ratio may help to distinguish AD from neuroinflammatory diseases. Further studies are needed to confirm these findings.

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AUTHOR CONTRIBUTIONS

PS, RL, UK, PL, NE, and JM designed the study. PS, JK, TO, PL, UK, HH, and JM investigated the patients and collected the samples. PS, PL, TO, and NE carried out the experiments. The statistics were carried out by PS. PS and JM drafted the manuscript. All authors critically reviewed the manuscript and provided constructive comments to improve the quality of the manuscript. All authors have read and approved the final manuscript.

ACKNOWLEDGMENTS

This work is supported by grants from the Interdisciplinary Center for Clinical Research (IZKF), Erlangen. The research leading to these results also received support from the Innovative Medicines Initiative Joint Undertaking under EMIF grant agreement no. 115372, resources of which are composed of financial contribution from the European Union's Seventh Framework Program (FP7/2007–2013) and EFPIA companies' kind contributions. We acknowledge support from Deutsche Forschungsgemeinschaft (DFG) and Friedrich-Alexander-Universität Erlangen-Nürnberg (FAU) within the funding program Open Access Publishing.

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Conflict of Interest Statement: PL received consultation and lectures honoraria from Innogenetics, IBL International, AJ Roboscreen, and Roche.

The other authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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