

**Evaluation of Intrathecal Serum Amyloid P (SAP) and C-Reactive Protein (CRP) Synthesis
in Alzheimer's Disease with the Use of Index Values**

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ABSTRACT

Serum amyloid P (SAP) and C-reactive protein (CRP) are proteins involved in innate immunity. The expression of SAP and CRP is increased in Alzheimer's disease (AD) brain tissue, compared to healthy controls. Although both proteins are found in cerebrospinal fluid (CSF), their origin is unclear. We investigated if increased local production of SAP and CRP in AD brain results in higher levels in CSF with the use of index values. To study this, SAP, CRP, and albumin levels were determined in CSF and serum samples of 30 control (65 ± 11 years; 57% female) and 140 AD subjects (65 ± 9 years; 53% female). To correct for inter-individual differences in protein diffusion from blood to CSF, quotients ($Q = \text{CSF}/\text{serum}$) of SAP, CRP, and albumin and index values ($Q_{\text{protein}}/Q_{\text{alb}}$) were calculated. The results showed no significant differences in SAP and CRP index values between control and AD subjects, although eight percent of individual AD patients showed evidence of intrathecal SAP or CRP production using the Reiber hyperbolic model. Interestingly, the SAP index value was much lower than expected, based on its molecular size. In conclusion, these data suggest that local production of SAP and CRP in the AD brain does not substantially contribute to the CSF levels.

Keywords: Alzheimer's disease, C-reactive protein, index value, serum amyloid p

INTRODUCTION

Serum amyloid P (SAP) and C-reactive protein (CRP) both belong to the family of pentraxins, which are highly conserved during evolution. SAP and CRP share 51% homology in amino acid sequence [1]. In serum, both proteins are made up of five non-covalently bound identical monomers, which are arranged in a flat pentameric disk [2,3].

SAP and CRP are pattern recognition molecules that can interact with a variety of ligands. CRP can bind to microorganisms, apoptotic cells, and modified low-density lipoproteins, whereas SAP can bind to bacteria, apoptotic cells, nuclear materials, and amyloid fibrils. C1q, the recognition unit of the complement system, can bind to SAP and CRP, aggregated or bound to their ligands, and initiate complement activation [4-7], which suggests that the complement system and pentraxins tightly cooperate as key components of the humoral arm of the innate immune system.

Alzheimer's disease (AD) is the most common form of dementia and immunohistochemically characterized by the presence of amyloid plaques and tau tangles. Amyloid plaques amongst others contain SAP, which is believed to be involved in the formation of amyloid plaques [8,9]. Because SAP is resistant against proteolysis, its binding to amyloid fibrils, may hamper the removal of amyloid deposits by preventing their proteolytic breakdown [10]. SAP is a constitutive serum protein, whereas CRP acts as an acute phase protein, of which serum levels rapidly rise upon inflammation and infection. Although both SAP and CRP are mainly synthesized by the liver, these proteins are also locally produced in brain tissue [11]. Interestingly, increased mRNA and protein levels of SAP and CRP were detected in brain of AD patients compared to controls [11,12], suggesting involvement of both pentraxins in AD pathogenesis.

Proteins in CSF may originate from local production in the brain and from diffusion from the plasma compartment. Brain derived proteins in CSF are thought to reflect specific (pathological) processes in the brain, whereas diffusion of plasma proteins into the CSF is mainly dependent on their size, as well as on the integrity of the blood-CSF-barrier. CSF levels alone are not informative for the origin of CSF proteins. Therefore, an index value representing the CSF concentration of a protein divided by its serum concentration and in turn divided by the CSF/serum quotient of a protein normally not passing the blood-CSF-barrier, such as albumin, can be used to discriminate between a blood-derived and a pathological brain specific protein fraction in CSF and takes into account individual changes in blood-CSF-barrier function. The use of index values has been proven to be an efficient way to determine intrathecal IgG synthesis in multiple sclerosis patients [13] and intrathecal C4 synthesis in patients with different infectious diseases of the central nervous system [14]. Thus, the index value of a protein can be used to determine the contribution of intrathecal synthesis to its CSF level, but could also serve as a specific brain pathology biomarker. Neuroinflammation plays an important role in AD pathology and increased production of SAP and CRP in AD brain has been described [11]. Therefore, we hypothesize that AD patients will have increased SAP and CRP indexes compared to controls.

MATERIALS AND METHODS

Patients

We included 140 AD patients and 30 control subjects in this study. All patients underwent a standardized clinical investigation including medical history, physical, and neurological examination, screening laboratory tests, and magnetic resonance imaging. The clinical diagnoses were made in conference by a multidisciplinary team, without knowledge of CSF data. Only AD

cases who fulfilled the NINCDS-ADRDA criteria of probable AD were included [15]. The control group consisted of 14 patients who presented at our memory clinic, but had normal results in all clinical and neuropsychological examinations. These cases were considered to have subjective memory complaints. Additionally, 16 volunteers without cognitive complaints, who were willing to undergo the same diagnostic procedure as patients attending our memory clinic, were included as control subjects. The local ethical review board approved the study protocol and all subjects gave written informed consent.

CSF and serum analyses

CSF samples were obtained by lumbar puncture between the L3 and L4 or L4 and L5 intervertebral space and collected in polypropylene tubes. A sample of blood was obtained at the same time. A small amount of CSF was used for routine analysis, including cell count and total protein determination. Both CSF and serum samples were centrifuged at 1800 g for 10 min (4°C). CSF and serum samples were aliquoted into polypropylene tubes and immediately stored at -80°C until analysis.

CSF and serum albumin concentrations were determined by nephelometry in a Beckman Coulter Immage 800 immunochemistry system. The lower detection limit of albumin in serum is 0.22 g/L. Intra assay variation with internal controls with average values of 22.5 and 49.0 g/L were 4.0% (n=306) and 4.3% (n=304), respectively. Serum and CSF samples were tested in the same run and results were expressed as mg/L for CSF and g/L for serum. Subsequently, the CSF/serum quotient of albumin was calculated to assess the intactness of blood-CSF-barrier function. An upper reference limit of Q_{alb} was $10 \cdot 10^{-3}$ was used [16].

Levels of CRP were determined by a semi-automated ELISA method on a CODA automated EIA reader (Bio-Rad) and levels of SAP by a manual ELISA method as described before [17,18]. The lower detection limit is 0.1 µg/l for both the SAP and CRP assay. The intra-assay coefficient of variance was 3.2% (n=14) for SAP and 3.9% (n=21) for CRP. The inter-assay coefficient of variance was 13.1% for SAP (n=35; period from 2005 to 2008) as determined for three aliquoted pools and 6.7% (n=62) and 7.1% (n=59) for CRP low (0.76 mg/L) and high (7.49 mg/L) pools respectively. All pools were stored at -80°C and run each time as internal control.

CSF A β ₄₂, t-tau, and p-tau-181 were determined by commercially available ELISAs (Innogenetics, Ghent, Belgium) as described before [19-21]. The team involved in the CSF analysis was not aware of the clinical diagnoses. As the manufacturer does not supply control data, performance of the assays was monitored using pools of surplus CSF specimens including multiple specimens with various concentrations in 7-18 runs. The inter-assay coefficient of variation was 11.3% \pm 4.9% for A β ₄₂, 9.3% \pm 1.5% for t-tau, and 9.4% \pm 2.5% for p-tau-181.

The quotient of a protein was calculated using the following equations: Quotient (Q) of a protein = CSF concentration/serum concentration. The use of the albumin CSF/serum quotient (Q_{alb}) has been shown to be a well established marker for individual blood-CSF-barrier function [16,22], therefore the index value of a protein is described as Q_{protein}/ Q_{alb}. Individual intrathecal synthesis of SAP and CRP was evaluated according to the method described by Reiber [23-25], by using an adapted version of the Reiber hyperbolic model. Therefore, Q_{SAP} and Q_{CRP} were plotted against Q_{alb} for control subjects only, to determine the reference ranges (mean \pm 3 SD) of SAP and CRP in CSF (Fig 2).

Statistical analysis

All analyses were performed using SPSS version 15.0 for Windows (SPSS Inc, Chicago, USA). Levels of CSF and serum markers were compared between controls and AD subjects using the non-parametric Mann-Whitney U test and levels were depicted as median [range] values. The SAP index and CRP index were compared using the Wilcoxon signed Ranks test. Spearman correlation was used to investigate the relation of Q_{alb} with Q_{SAP} and Q_{CRP} . A p value of ≤ 0.05 was considered to reflect statistical significance.

RESULTS

Levels of SAP, CRP, and albumin, that were determined in paired CSF and serum samples of control and AD subjects, are shown in Table 1. No differences were found for Q_{SAP} and Q_{CRP} between controls and AD patients. When the quotients were corrected for blood-CSF-barrier changes, also no significant differences between control subjects and AD cases were found for both the CRP index ($p=0.70$) and the SAP index ($p=0.10$; Figure 1). Individual index values can be used to determine the contribution of intrathecal production to the CSF levels of individual AD patients. Using the Reiber hyperbolic model, only six out of 137 (4%) AD patients showed intrathecal synthesis of SAP and in six other patients intrathecal synthesis of CRP was found (Figure 2).

High correlations between Q_{alb} and Q_{SAP} (controls: $r=0.84$; AD: $r=0.76$ both $p<0.01$), Q_{alb} and Q_{CRP} (controls: $r=0.81$; AD: $r=0.53$ both $p<0.01$) were found. This was also observed between CSF SAP and serum SAP (controls: $r=0.56$; AD: $r=0.48$ both $p<0.01$), and CSF CRP and serum CRP (controls: $r=0.92$; AD: $r=0.83$ both $p<0.01$).

Both SAP and CRP proteins have the same molecular mass, therefore comparable values of quotients and indexes might be expected. However, an eight times lower SAP index compared to the index for CRP ($p \leq 0.001$) was detected in the entire group (7.5 [0.4–359]). When calculated for each diagnosis separately, controls had a six times lower SAP index compared to their CRP index, whereas in AD patients this difference was eight fold (controls: 6.4 [3.6–13.0]; AD: 7.6 [0.4 – 359], $p=0.08$).

DISCUSSION

This study shows that the increased production of SAP and CRP in the AD brain does not appreciably contribute to their CSF levels. Furthermore, we found that the index value of SAP was six to eight times lower than that for CRP, suggesting limited diffusion of SAP from the brain to CSF, but more likely limited diffusion from the blood to CSF compared to CRP.

Next to brain derived proteins in CSF, proteins can also be derived from the blood via passive diffusion across the blood-CSF-barrier. Index values can be used to determine differences in CSF levels (as a result of intrathecal synthesis), irrespective of blood levels and blood-CSF-barrier permeability as shown before for C4 and IgG [13,14]. Amyloid plaques in the brain consist of amyloid beta peptide and amyloid associated proteins [26]. Several studies have shown that SAP is a major constituent of the amyloid plaques [27,28], whereas CRP shows a more granular staining near plaques, suggesting staining of neuritic nerve endings [11,29]. Although increased SAP and CRP mRNA expression in AD brain has been reported [11], our results show no difference in SAP- and CRP index values between control cases and AD patients. Although the SAP index was not significantly different between control and AD patients ($p=0.1$), it does suggest a trend towards a reduced SAP index value in AD patients,

suggesting that despite increased synthesis, SAP levels could be reduced because of deposition in amyloid plaques. These findings are in line with the results of another study, in which SAP levels in CSF were shown to be lower in patients with mild cognitive impairment (MCI) that at follow up had progressed to AD, compared to MCI patients that remained stable [18].

On an individual level, only 8% of the AD patients showed increased intrathecal synthesis of either SAP or CRP in brain as concluded from the increased index values compared to controls. These results do not exclude local production in the brain, but for the AD group as a whole the contribution of protein being brain derived appears to be superseded by diffusion from blood as shown by the high correlations between Q_{alb} and both Q_{SAP} and Q_{CRP} .

The use of patients with subjective memory complaints in the control group could be considered a limitation of this study, since these cases might present the earliest signs of AD pathology. However, $A\beta_{42}$ levels in combination with tau and/or p-tau levels in all these cases were within the normal range [17,30]. In addition, no differences in Q_{SAP} and Q_{CRP} were found between patients with subjective complaints and healthy volunteers.

Furthermore, 30 control subjects compared to 140 AD patients were used in this study, which could possibly limit our statistical power. Increasing the number of control subjects could have possibly increased the power of the study; however this most likely will not affect the message of this study.

Protein diffusion from blood to CSF is mainly related to the size of a protein [31]. SAP and CRP in serum have been described as both having a pentameric structure with approximately the same molecular mass [3,32], which will likely result in comparable quotient and index values for these proteins. Here we show that the SAP index is six to eight times lower than the CRP index, suggesting limited diffusion of SAP from blood to CSF. Although there is some discrepancy in

literature whether SAP in serum is present as a pentamer, or as a decamer [3,32,33], this cannot explain the difference in index values. For example, SAP in a decameric form, would have a molecular mass of 250 kD [34,35], which is closely related to that of C4 (200 kD) with a corresponding index value of 0.74 in control subjects [14], which is much higher than we observed (SAP index controls: 0.06). IgM with a molecular mass of 900 kD, by contrast, has an index of 0.02 [36], which is comparable to that of SAP. Thus, the diffusion rate of SAP from blood to CSF is lower than can be expected based on its size alone. This is also illustrated by the shape of the Reibergrams of SAP and CRP in figure 2. The Reibergram for CRP shows a similar straight pattern as seen for IgG [25], whereas the Reibergram for SAP resembles a more “flat” pattern as seen for IgM [25], which has a higher molecular size than IgG. However, it is considered unlikely that SAP occurs in aggregated form in serum [3,32], therefore the very low index is probably not due to higher molecular weight SAP species in blood.

Except for molecular size, other factors, such as the iso-electric point of a protein, may also determine the diffusion of proteins across the blood-CSF-barrier. Membranes in general are negatively charged, and proteins with a negative charge will, therefore, encounter more resistance to diffuse across a membrane than cationic proteins. In contrast to CRP, each SAP subunit has been shown to contain two sialic acids [34], which could cause SAP to be slightly more negative resulting in a hampered diffusion of SAP through the blood-CSF-barrier compared to CRP. However, it is not very likely that this will cause the 6-8 fold difference in index values we observed. More research is obviously needed to provide more insight in the origin of the low levels of SAP in CSF.

Although the SAP and CRP index values are not suitable as a biomarker for AD, these findings illustrate the importance of not only detecting proteins in CSF, but also in serum and the necessity to correct for blood-CSF-barrier functioning.

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Table 1. Demographics, CSF, serum, quotients (Q) and index values for SAP and CRP in control and AD subjects

	Controls (n=30)	AD (n=140)
Age (y; mean \pm SD)	65 \pm 11	66 \pm 9
Gender (% female)	57	55
MMSE (mean \pm SD) ¹	29 \pm 1	21 \pm 5*
ApoE- ϵ 4 carrier (%) ²	15	73*
A β ₄₂ (pg./ml)	805 (494-1142)	384 (124-1320)
t-tau (pg/ml)	288 (129-608)	685 (75-2615)
p-tau (pg/ml)	46 (22-78)	83 (18-279)
SAP CSF (μ g/L)	15 (4-109)	14 (2-172)
SAP serum (mg/L)	50 (26-85)	48 (20-147)
CRP CSF (μ g/L)	3.58 (0.7-26.6)	2.35 (0.5-220)
CRP serum (mg/L)	1.23 (0.20-14.64)	0.91 (0.06-118)
Q _{SAP} ($\times 10^{-3}$)	0.32 (0.10-1.90)	0.31 (0.10-3.10)
Q _{CRP} ($\times 10^{-3}$)	2.2 (0.8-7.9)	2.6 (0.1-55.5)
Q _{alb} ($\times 10^{-3}$) ³	5.3 (2.0-19.7)	5.6 (2.6-16.3)
SAP index ³	0.06 (0.03-0.10)	0.05 (0.01-0.32)
CRP index ³	0.40 (0.23-0.76)	0.43 (0.02-14.3)

Q_{protein} = CSF concentration/serum concentration and Index = Q_{protein}/ Q_{alb}. Protein concentrations, quotients and indexes depicted as median (min – max).¹ five missing values in control subjects and 11 missing values in the AD patients group, ² three missing values in control subjects and 15 missing values in the AD patients group ³ three missing values in the AD patients group. * p<0.001

FIGURE LEGENDS

Figure 1. SAP index values for control and AD subjects. A trend towards a lower SAP index in AD patients compared to controls is observed ($p=0.10$).

Figure 2. Q_{SAP} (A and B) or Q_{CRP} (C and D) plotted against Q_{alb} gives insight in the individual intrathecal synthesis of SAP and/or CRP. Reference ranges are determined in the control group ($\text{mean} \pm 3SD$; A and C) and depicted as a hyperbolic function. The vertical dotted line indicates the upper reference limit for Q_{alb} at $10 \cdot 10^{-3}$. Open circles indicate control subjects (A,C) and filled circles AD patients (B,D). Values above the Q-max line in the AD CSF samples indicate intrathecal synthesis of either SAP (B) or CRP (C).

Figure 1

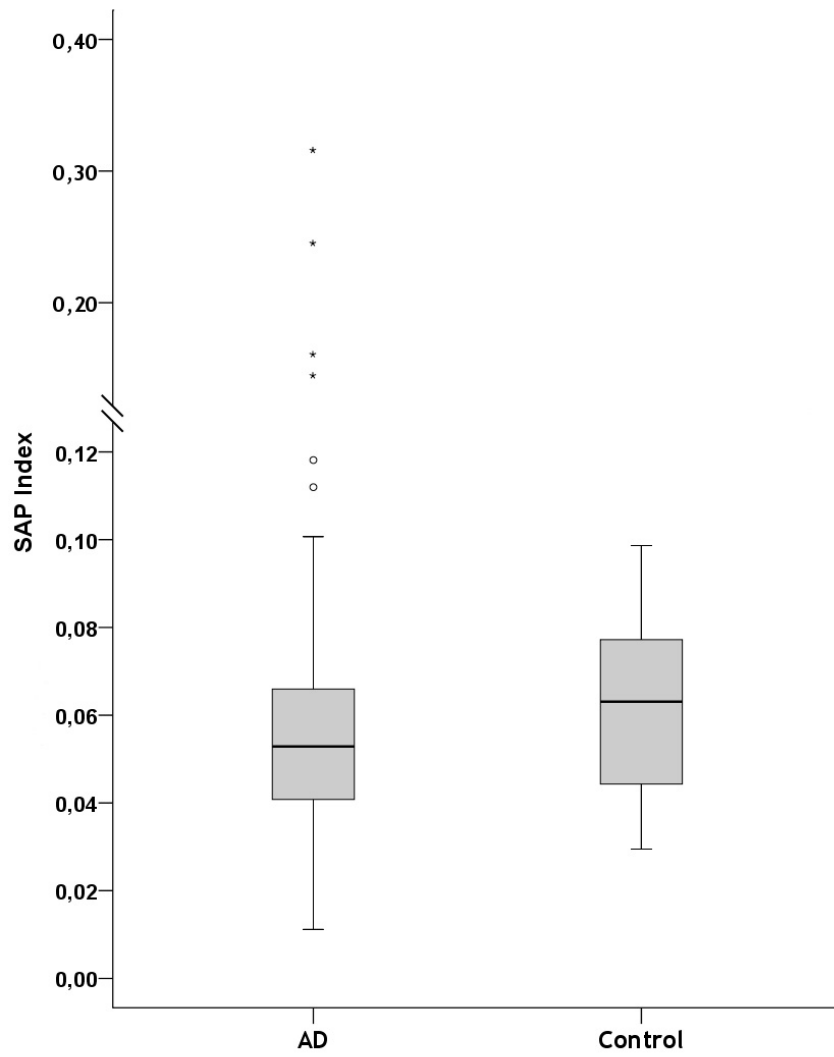


Figure 2

