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Research Report

Plasma Amyloid-β in Relation to Antibodies Against Herpes Simplex Virus, Cytomegalovirus, and *Chlamydophila pneumoniae*

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Abstract.
Background: Amyloid-β (Aβ), the key constituent of Alzheimer's disease (AD) plaques, has antimicrobial properties.
Objective: To investigate the association between plasma Aβ and antibodies against the AD-related pathogens herpes simplex virus (HSV), cytomegalovirus (CMV), and *C. pneumoniae*.
Methods: Plasma from 339 AD cases, obtained on average 9.4 years (± 4.00) before diagnosis, and their matched controls were analyzed for $A\beta_{40}$ and $A\beta_{42}$ concentrations with Luminex xMAP technology and INNOBIA plasma Aβ-form assays. Enzyme-linked immunosorbent assays were utilized for analyses of anti-HSV immunoglobulin (Ig) G, anti-HSV1 IgG, anti-HSV2 IgG, anti-CMV IgG, and anti-*C. pneumoniae* IgG. Follow-up samples were available for 150 of the cases.
Results: Presence and levels of anti-HSV1 IgG, anti-HSV2 IgG, anti-CMV IgG, and anti-*C. pneumoniae* IgG did not correlate with concentrations of $A\beta_{42}$ or $A\beta_{40}$ in cases or controls.
Conclusion: Levels of plasma Aβ were not associated with antibodies against different AD-related pathogens.

Keywords: Alzheimer's disease, amyloid-β peptides, *Chlamydophila pneumoniae,* cytomegalovirus, dementia, Herpes simplex, nested case-control study

INTRODUCTION

Alzheimer's disease (AD) is a terminal condition that affects millions of people worldwide. The etiology of sporadic AD is not completely understood, but a growing body of evidence points to an infectious component in AD development [1, 2]. Herpes simplex virus type 1 (HSV1), cytomegalovirus (CMV), and *Chlamydophila pneumoniae* are all pathogens that have been associated with AD [3–10].

HSV1 is ubiquitous and found in approximately 80–90% of the adult population [11]. After primary infection, the virus persists latently in sensory

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neurons, most commonly in the trigeminal ganglion, and can subsequently reactivate. HSV1 has been detected in a high proportion of brains from AD patients and co-localizes with amyloid plaques in particular [12–16]. In epidemiological studies, carrying HSV1 has been associated with increased risk of AD, especially among genetically susceptible individuals [3–7, 17–21]. Similar to HSV1, the seroprevalance of CMV is high, reaching 80–90% in adults [11, 22]. Following primary infection, the virus remains latent in T lymphocytes and can be reactivated repeatedly without causing symptoms [23]. Co-infection of CMV and HSV1 has been reported to increase the risk of developing AD [24, 25]. *C. pneumoniae* is a bacterium that can cause respiratory symptoms. Asymptomatic infection also occurs and approximately 60% of the adult population has serological evidence of *C. pneumoniae* infection [26].

The hypothesis of infectious involvement in AD corresponds well with the finding that amyloid-β (Aβ) has potent antimicrobial activity [27–29]. Cultured cells accumulate Aβ when infected with a broad spectrum of viruses (including HSV1 and CMV), bacteria, and fungi [9, 27, 29–33]. In addition, both HSV1 and *C. pneumoniae* infection lead to the formation of amyloid plaques in murine models [28, 29, 34–36]. Mice overexpressing Aβ have greater survival rates compared to non-transgenic mice after brain infection [28, 29], suggesting a potential link between high levels of Aβ and resistance to infection.

Aβ is primarily produced in the brain. However, there are potential extracerebral sources of Aβ, such as platelets [37, 38] and fibroblasts [9]. Aβ containing 40 amino acids ($A\beta_{40}$) is the most common variant of the Aβ peptide in the central nervous system. The longer $A\beta_{42}$ fragment is the most amyloid-prone and is thus considered more neurotoxic than $A\beta_{40}$ [39]. Soluble plasma concentrations of both variants might vary as a consequence of pathogens infecting peripheral tissues. A previously published study showed that increased levels of anti-HSV IgM were associated with lower plasma levels of both $A\beta_{40}$ and $A\beta_{42}$ [40]. The correlation between plasma Aβ levels and antibodies against CMV and *C. pneumoniae* has not been previously investigated.

Relationships between $A\beta_{40}$ and $A\beta_{42}$ in plasma and antibodies against different pathogens might indirectly support earlier findings that Aβ has antimicrobial properties. Therefore, the aim of this study was to analyze the potential associations between plasma Aβ levels and circulating antibodies against HSV, CMV, and *C. pneumoniae*.

METHODS

Participants and procedures

The nested case-control study used plasma samples previously donated and stored in the Medical Biobank in Umeå (Northern Sweden Health and Disease Study) [41]. A computerized procedure was employed to select AD cases with a suitable matched control. We identified 339 pairs of AD cases and matched controls who had been analyzed for Aβ. Controls were matched by sampling date, age, sex, and cohort from the Biobank material. In addition, follow-up samples were obtained for 150 of the 339 AD cases, collected between the time of the first sample and the date of diagnosis (ranging from 1 to 5 additional samples per subject, mean 1.59 samples ± 0.84, with a total of 251 follow-up samples). For this particular sample, analysis of Aβ concentrations and the risk of AD development has previously been published [42]. The study was approved by the Regional Ethical Review Board in Umeå, Sweden (09–190M).

AD diagnosis

AD cases were diagnosed at the Memory Clinic of the University Hospital in Umeå according to the criteria of The Diagnostic and Statistical Manual of Mental Disorders, fourth edition [43], with a minimum of one brain-imaging technique. The clinical AD diagnoses were compatible with the National Institute of Neurological and Communicative Disorders and Stroke—Alzheimer's Disease and Related Disorders Association criteria [44]. Controls were confirmed free from major neurocognitive disorders and alive at the time of diagnosis for their corresponding AD patient using nation-wide registries. This procedure is described in detail in a previous publication [5].

Plasma analysis

$A\beta_{40}$ and $A\beta_{42}$ concentrations were determined with Luminex xMAP technology and the INNOBIA plasma Aβ-form assays (Innogenetics, Ghent, Belgium) [45]. In-house enzyme-linked immunosorbent assays (ELISA) were used for the analysis of anti-HSV IgG and anti-CMV IgG antibodies, as described in a previous publication [46]. Commercial ELISA kits were utilized for the analysis of anti-HSV1 IgG, anti-HSV2 IgG (HerpeSelect 1, HerpeSelect 2,

FOCUS Diagnostics), and anti-*C. pneumoniae* (SeropCp™ Quant IgG).

To establish carriage of HSV1, HSV2, or both, each anti-HSV IgG-positive sample was further analyzed for anti-HSV2 IgG. If positive, an additional analysis was conducted for anti-HSV1 IgG to differentiate between HSV1 positive, HSV2 positive, and HSV1 + HSV2-positive individuals.

Statistical analysis

Linear regression analyses of Aβ and antibodies against HSV, HSV1, HSV2, CMV, and *C. pneumoniae* were performed separately in groups of cases and controls. Age and sex were included in the linear regression models as potential confounders, if they were significantly associated with both the dependent and the independent variables in that particular regression. Pearson's correlation, Student's *t*-test, the chi-squared test, and Fischer's exact test were used for these analyses as appropriate.

The paired-sample *t*-test and McNemar's test were used to compare differences between the matched groups concerning the presence of anti-HSV, anti-HSV1 IgG, anti-HSV2 IgG, anti-CMV, and anti-*C. pneumoniae* IgG antibodies.

Linear mixed models were fitted to analyze Aβ plasma concentrations over time and seropositivity for different antibodies among AD patients with repeated samples. In this model, time was defined as the number of years between the sampling and diagnosis date. The interactions between sex and age with time were considered potential confounders if significantly associated with antibody seropositivity. The models were performed in R version 3.4.3 using the "lme4 package" and included a subject-specific random intercept to account for within-subject correlations between repeated measurements.

The assumptions of the linear mixed and regression models were tested before carrying out the analyses. Aβ was plotted against follow-up time and IgG levels to visualize their relationship. Normality was evaluated with Q-Q plots and simple histograms of the distribution of Aβ in different groups. Levene's test were used to assess the homogeneity of variance.

SPSS Statistics version 24 (IBM Corporation, Armonk, NY) was used for statistical analyses, with a p-value of < 0.05 being interpreted as statistically significant. p values were adjusted for multiple comparisons by the Bonferroni correction method ($n = 18$ analyses for $A\beta_{40}$ and $A\beta_{42}$ respectively).

RESULTS

The study enrolled 339 cases with AD and 339 matched controls (76.4% females). The mean age at plasma sampling was 61.3 years in both groups and the mean age at diagnosis was 70.8 years (Table 1). AD cases had significantly higher seroprevalence of anti-HSV IgG compared to their controls (94.4% versus 89.7%, respectively, $p = 0.030$; Table 1). Otherwise, no differences between the groups were observed in terms of Aβ plasma concentrations and the presence of anti-HSV1 IgG, anti-HSV2 IgG, anti-CMV IgG, or anti-*C. pneumoniae* IgG antibodies (Table 1).

Table 1
Basic characteristics of the sample

	AD cases $n = 339$	Controls $n = 339$	AD cases with repeated samples $n = 150$[1]	p for differences between cases and controls[2]
Age at sampling (y), mean ± SD	61.3 ± 5.6	61.3 ± 5.6	59.8 ± 5.5	
Age at diagnosis (y), mean ± SD	70.8 ± 6.4		70.3 ± 6.3	
$A\beta_{42}$ (ng/L), mean ± SD	43.6 ± 13.1	44.6 ± 12.5	43.5 ± 12.5	0.316
$A\beta_{40}$ (ng/L), mean ± SD	142.3 ± 36.3	143.9 ± 41.0	140.9 ± 37.9	0.525
Sex (female), n (%)	259 (76.4)	259 (76.4)	141 (94.0)	
anti-HSV IgG+, n (%)[3]	320 (94.4)	304 (89.7)	143 (95.3)	0.030
anti-HSV1 IgG+, n (%)[3]	311 (91.7)	297 (87.6)	142 (94.7)	0.075
anti-HSV2 IgG+, n (%)[3]	49 (14.5)	43 (12.7)	14 (9.3)	0.450
anti-CMV IgG+, n (%)	293 (86.4)	299 (88.2)	135 (90.0)	0.485
anti-*C. pneumoniae* IgG+, n (%)	209 (61.7)	205 (60.5)	88 (58.7)	0.737
anti-HSV IgM+, n (%)	24 (7.1)	18 (11.8)	14 (9.3)	0.411
anti-CMV IgM+, n (%)	10 (2.9)	12 (3.5)	4 (2.7)	0.491

AD, Alzheimer's disease; y, years; SD, standard deviation; Aβ, amyloid-β; HSV, herpes simplex virus; Ig, immunoglobulin; CMV, cytomegalovirus; [1]At baseline. [2]Comparison between "AD cases, $n = 339$" and "Controls, $n = 339$". [3]40 subjects (11.8 %) were positive for both anti-HSV1 and anti-HSV2 IgG among the cases, and 18 (5.3 %) subjects were positive for both anti-HSV1 and anti-HSV2 IgG among the controls, and 13 (8.7 %) subjects were positive for anti-HSV1 and anti-HSV2 IgG among the cases with repeated samples.

Table 2
Simple linear regression with plasma concentrations of free Aβ in relation to seropositivity for antibodies (indicating carriage of a pathogen)

	$A\beta_{42}$				$A\beta_{40}$			
	AD cases		Controls		AD cases		Controls	
	β	p, $p_{adj.}$[1]	β	p, $p_{adj.}$[1]	β	p, $p_{adj.}$[1]	β	p, $p_{adj.}$[1]
anti-HSV1 IgG+	–0.018	0.735, 1.000	0.085	0.117, 1.000	–0.039	0.479, 1.000	–0.029	0.599, 1.000
anti-HSV2 IgG+	0.079	0.145, 1.000	–0.039	0.476, 1.000	0.132	0.015, 0.270	–0.013	0.805, 1.000
anti-CMV IgG+	0.018	0.743, 1.000	–0.013	0.806, 1.000	–0.068[2]	0.210, 1.000	–0.001	0.980, 1.000
anti-*C. pneumoniae* IgG+	–0.042	0.438, 1.000	0.060	0.271, 1.000	0.014	0.791, 1.000	–0.113	0.038, 0.684

Aβ, amyloid-β; AD, Alzheimer's disease; HSV, herpes simplex virus; Ig, immunoglobulin; CMV, cytomegalovirus; *C. pneumoniae, Chlamydophila pneumonia;* [1]Adjusted for multiple comparisons by Bonferroni correction, $n = 18$ analyses for $A\beta_{40}$ and $A\beta_{42}$ respectively. [2]Adjusted for sex.

Table 3
Simple linear regression with plasma concentrations of free Aβ in relation to levels of antibodies

	$A\beta_{42}$				$A\beta_{40}$			
	AD cases		Controls		AD cases		Controls	
	β	p, $p_{adj.}$[1]	β	p, $p_{adj.}$[1]	β	p, $p_{adj.}$[1]	β	p, $p_{adj.}$[1]
anti-HSV IgG levels[1]	0.016	0.769, 1.000	–0.087	0.130, 1.000	0.090	0.736, 1.000	0.081	0.157, 1.000
anti-CMV IgG levels[1]	–0.034	0.567, 1.000	–0.127	0.028, 0.504	–0.019[2]	0.751, 1.000	–0.088	0.127, 1.000
anti-*C. pneumoniae* IgG levels[1]	–0.063	0.363, 1.000	0.004	0.951, 1.000	0.053	0.449, 1.000	0.097	0.168, 1.000

Aβ, amyloid-β; AD; Alzheimer's disease; HSV, herpes simplex virus; Ig, immunoglobulin; CMV, cytomegalovirus; *C. pneumoniae,* [1]Adjusted for multiple comparisons by Bonferroni correction, $n = 18$ analyses for $A\beta_{40}$ and $A\beta_{42}$ respectively. [2]Adjusted for sex.

Table 4
Mixed linear model with the interaction between follow-up time and seropositivity on Aβ plasma concentrations among individuals with Alzheimer's disease

	$A\beta_{42}$		$A\beta_{40}$	
Interaction with follow-up time	Estimate[1] ± SE	p, $p_{adj.}$[2]	Estimate±SE	p, $p_{adj.}$[2]
anti-HSV1 IgG+ × time[3]	–0.582 ± 0.507	0.251, 1.000	0.426 ± 1.717	0.804, 1.000
anti-HSV2 IgG+ × time[3]	0.150 ± 0.415	0.718, 1.000	–0.517 ± 1.327	0.697, 1.000
anti-CMV IgG+ × time[3]	–0.241 ± 0.4107	0.558, 1.000	–1.424 ± 1.384	0.304, 1.000
anti-*C. pneumoniae* IgG+ × time[3]	0.398 ± 0.254	0.119, 1.000	–1.997 ± 0.844	0.018, 0.324

Aβ, amyloid-β; HSV, herpes simplex virus; Ig, immunoglobulin; CMV, cytomegalovirus; [1]Estimated yearly change in nanograms/liter. [2]Adjusted for multiple comparisons by Bonferroni correction, $n = 18$ analyses for $A\beta_{40}$ and $A\beta_{42}$ respectively. [3]The analysis included subjects with AD and repeated measurements (i.e., follow-up samples), number = 163.

Neither $A\beta_{42}$ nor $A\beta_{40}$ levels were, after adjustment for multiple comparisons, associated with presence or levels of anti-HSV1 IgG, anti-HSV2 IgG, anti-CMV IgG and anti-*C. pneumoniae* IgG (Tables 2 and 3).

Plasma concentrations of $A\beta_{42}$ and $A\beta_{40}$ in relation to follow-up time among AD patients were not affected by age ($p = 0.367$ and $p = 0.911$, respectively) or sex ($p = 0.618$ and $p = 0.997$, respectively). There were no significant interactions between follow-up time and the presence of anti-HSV1 IgG, anti-HSV2 IgG, anti-CMV IgG, and anti-*C. pneumoniae* IgG in association with $A\beta_{42}$ and $A\beta_{40}$ after adjustment for multiple comparisons (Table 4).

DISCUSSION

This nested case-control study included a large number of thoroughly diagnosed AD cases and closely matched controls, with plasma samples taken on average almost 10 years prior to diagnosis. The study material was therefore well suited to investigate whether biochemical markers for AD correlate with

infectious agents. Here, we specifically investigated the prevalence and titers of IgG towards possible AD-related pathogens and the concentrations of free, soluble $A\beta_{42}$ and $A\beta_{40}$.

We found that Aβ concentrations did not correlate with antibodies against the different pathogens investigated. While the unadjusted analyses indicated a few associations, no clear patter emerged. None of these associations were significant after adjustment for multiple comparisons. Our negative results, therefore, do not indicate that Aβ is directly involved in managing AD-associated infections in the periphery. An early hypothesis was that the preclinical AD patients would react differently concerning Aβ concentrations in response to infection, possibly revealing altered ability to control peripheral infections, but no clear pattern was observed as to differences in the associations between AD cases and their controls. Importantly, we measured IgG antibodies, which can be considered markers of latent or chronic infection. Presence of IgG directed towards a specific pathogen could indicate carriage, while higher IgG levels could possibly reflect a more active or recently triggered immune response. Latent HSV1 is mainly localized to the trigeminal ganglia. At this locale, non-reactivating virus may not be exposed to the peripheral immune system, which could explain the lack of an association between anti-HSV1 IgG and plasma Aβ. It should also be noted that concentrations of free plasma Aβ do not necessarily correlate with concentrations of Aβ in the central nervous system and our results are not representative of intracerebral processes [42].

In the present study, AD cases had a significantly higher proportion of HSV seropositives compared to controls. This corresponds well with the growing evidence for an association between HSV1 and AD development [1, 3].

A limitation of the study is the small number of anti-HSV IgM seropositive individuals, making it difficult to investigate whether IgM antibodies might correlate with Aβ concentrations, an association which has been detected in a previous study [40]. Another limitation was that follow-up samples were available only for a subset of the AD cases, making it impossible to compare the groups of cases and controls with respect to the presence of antibodies and Aβ concentrations over time.

In conclusion, this large nested case-control study indicates that plasma Aβ concentrations are not associated with antibodies against AD-related pathogens (viruses and bacteria). Investigations of other cohorts will be needed to confirm the findings of this study as well as to determine the predictive value of serum biomarkers for later AD development.

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CONFLICT OF INTEREST

The authors have no conflict of interest to report.

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