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Exploring the role of multi-pathogen infections in Alzheimer's disease: A case–control study

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ABSTRACT

Background: Alzheimer's disease (AD) is a leading cause of dementia. Following the discovery of the amyloid's antimicrobial properties, research has focused on infectious agents as potential contributors. This study investigates the role of multi-pathogen infections in AD by analyzing serological and molecular microbiologic markers in serum and cerebrospinal fluid (CSF).

Methods: A unicentric case-control study was conducted in AD patients (n=44) and healthy controls (n=35) in a Spanish reference cognitive disorders unit. Serum and CSF samples were analyzed using chemiluminescence immunoassay and ELISA for several pathogens. Molecular detection was performed through nucleic acid amplification. Multivariable logistic regression with bootstrap validation identified independent predictors of AD.

Results: Seropositivity for HCMV and Coxiella burnetii was significantly more frequent in AD patients, with C. burnetii linked to rapid progression. No microbial DNA was detected in CSF. Age (OR 1.55, 95% CI 1.17–2.03), HCMV (OR 20.02, 95% CI 2.64–183.38), and Chlamydia pneumoniae (OR 8.60, 95% CI 0.96–96.57) seropositivity were independent predictors of AD in the adjusted logit model, with an AUC of 0.79 after bootstrap validation. Wide confidence intervals indicate uncertainty in the exact strength of association. Triple-positive patients (HCMV, C. pneumoniae, C. burnetii) showed faster progression, poorer neuropsychological performance, and impaired CSF biomarkers, including lower A40/A42 ratios and higher neurofilament light levels.

Conclusions: Latent infections with HCMV, C. pneumoniae, and C. burnetii may contribute to AD pathophysiology, possibly through systemic or immune-mediated mechanisms. Further studies are needed to replicate these findings and clarify the role of infectious burden in AD.

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Introduction

Alzheimer's disease (AD) is the leading cause of dementia and a major global health concern, affecting approximately 10% of individuals over the age of 65, with incidence rising with age [1]. Its pathological hallmarks include the accumulation of beta-amyloid (Aβ) plaques and neurofibrillary tangles (NFTs) composed of hyperphosphorylated tau protein, which together drive progressive neuronal degeneration, leading to memory loss and cognitive decline [2]. Although Aβ42 plaques have long been implicated in initiating these pathological processes, their exact role remains under scrutiny, particularly given the failure of Aβ-targeting therapies to halt disease progression [3]. This has shifted the focus of AD research toward exploring alternative pathological mechanisms, including neuroinflammation and its relation with microbial agents, oxidative stress, and immune dysfunction.

Neuroinflammation has emerged as a central contributor in AD, marked by dysregulation of inflammatory pathways, chronic glial activation, oxidative stress, and increased cytokine release – including reactive oxygen species, TGF-β1, and S100B – which exacerbate Aβ and tau pathology and impair neuronal homeostasis [4,5]. In recent years, a growing body of evidence suggests that microbial agents may act as upstream inducers of this inflammatory cascade. Intriguingly, Aβ appears to function as an antimicrobial peptide, capable of disrupting microbial membranes, agglutinating pathogens, and forming protective fibrils [6–8]. This has led to the hypothesis that Aβ deposition may represent an innate immune response to chronic or

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latent infection – initially protective, but ultimately pathogenic if sustained, leading to plaque accumulation, neuroinflammation, and subsequent neuronal damage [9].

Several pathogens, including herpesviruses, *Chlamydia pneumoniae*, and periodontal bacteria, have been linked to AD pathology [9–12]. Beyond isolated infections, recent studies have proposed that microbial synergy and co-infection may amplify neuroinflammatory signaling, promote biofilm formation, and enhance Aβ aggregation [12]. These processes may foster both systemic and CNS-specific immune dysregulation, potentially through mechanisms such as molecular mimicry or glial priming.

The aim of this study was to investigate the multivariable relationship between AD and various infectious agents potentially implicated in its onset or progression by analyzing serological and molecular microbiologic markers in both serum and cerebrospinal fluid (CSF) through a case–control methodology.

Methods

Study design and patient inclusion

A unicentric cross-sectional retrospective case–control study was conducted at the University and Polytechnic Hospital La Fe (Valencia, Spain). Patients under clinical follow-up at the Reference Unit of Cognitive Disorders of the Department of Neurology for suspected cognitive impairment between January 2020 and July 2023 were considered eligible.

Cases were defined as patients aged 50–80 years diagnosed with AD according to the criteria established by the National Institute on Aging and the Alzheimer's Association [13], including CSF biomarkers and neuropsychological evaluation, who did not meet the following exclusion criteria: coexistence of another major neurological pathology except mild cerebrovascular disease, coexistence of a major psychiatric disorder, severe leukoaraiosis determined by Fazekas grade III classification on magnetic resonance imaging (MRI), and a history of human immunodeficiency virus (HIV) infection, neurosyphilis, Whipple's disease, Lyme disease, or viral encephalitis.

Controls were defined as individuals aged 50–80 years with normal biomarkers in CSF, normal neuropsychological evaluation results, no evidence of cognitive impairment, and no history of delirium, who did not meet the same exclusion criteria as the cases. Patient selection for inclusion in the screening was performed randomly from a database of all eligible patients who had been referred to the Reference Unit using a random number generation system. All eligible control patients in the study period were finally included. A minimum sample size of 32 patients per group was estimated. This calculation assumed a mean seroprevalence of 70% for common pathogens and aimed to detect a 25% difference between the two groups, with 80% potency and a significance level of 0.05.

All patients gave written informed consent for the use of their biological samples. The study protocol was approved by the Ethics Committee of the Health Research Institute La Fe with registration code 2023/181–1 and was compliant with the Declaration of Helsinki.

Clinical and epidemiological variables

Demographic, epidemiological, and clinical parameters were recorded for each patient, which included age and date of birth, sex, educational level (primary, secondary, and/or intermediate vocational training, or higher education), toxic habits (smoking, alcohol consumption, history of drug abuse), urban or rural residence, and socioeconomic status (high, middle, low). Additionally, comorbidities and concomitant medication were registered, including medical history (hypertension, dyslipidemia, diabetes mellitus, cerebrovascular disease, chronic ischemic heart disease, autoimmune or rheumatologic disorders, chronic kidney disease stage IIIa or higher) and concomitant medication use (statins, antiepileptics, antidepressants, antihypertensives, corticosteroids, anti-inflammatories, and immunosuppressants). The dates of Alzheimer's disease symptom onset and definite diagnosis were also documented.

AD biomarkers and neuropsychological assessment

Alzheimer's disease biomarkers Aβ1–40, Aβ1–42, t-Tau, p-Tau181, and neurofilament light (NfL) were determined in CSF samples by CLIA according to manufacturer instructions (Lumipulse® G, Fujirebio). Aβ40 and Aβ42 ratio and t-Tau and Aβ42 ratio were calculated. Apolipoprotein E (APOE) genotype (E2, E3, and E4 alleles) was determined by real-time PCR.

The neuropsychological assessment was performed by accredited neuropsychologists using the Clinical Dementia Rating (CDR) scale, the Mini-Mental State Examination (MMSE), and the Repeatable Battery for the Assessment of Neuropsychological Status (RBANS).

For the AD patients ($n = 44$), two neuropsychological evaluations at different moments of time were registered to define two progression categories:

rapidly progressing AD and non-rapidly progressing AD. Rapid progression was identified by a decline of 3 points on the MMSE within 1 year and/or an increase of 1.5 points on the CDR scale within 2 years.

Serological and molecular microbiologic analysis

Serum and CSF samples were collected during routine clinical practice for diagnosis, processed within 30 minutes, and stored at −80°C until analysis. Serum samples were tested by chemiluminescence immunoassay (CLIA) according to the manufacturer's instructions for IgG antibodies against HCMV (LIAISON® CMV IgG II by, DiaSorin), viral capsid antigen of EBV (LIAISON® VCA IgG, DiaSorin), HSV-1 (LIAISON® HSV-1 Type Specific IgG, DiaSorin), *Mycoplasma pneumoniae* (LIAISON® *Mycoplasma pneumoniae* IgG, DiaSorin), *Toxoplasma gondii* (LIAISON® Toxo IgG II, DiaSorin), VZV (LIAISON® VZV IgG, DiaSorin), *Chlamydia pneumoniae* (*Chlamydia pneumoniae* VIRCLIA® IgG Monotest, Vircell, S.L.), phase II antigens of *Coxiella burnetii* (*Coxiella burnetii* VIRCLIA® IgG Monotest, Vircell, S.L.); and by enzyme-linked immunosorbent assay (ELISA) for IgG against *Toxocara canis* with *T. cati* cross-reactivity (NovaLisa TM, NovaTec Immundiagnostica GmbH). Furthermore, IgG against *Toxocara* spp. were also determined in CSF samples with the same methodology. All determinations were blinded and performed in duplicate.

Additionally, all CSF samples were screened for the molecular presence of pathogens with neurological tropism using nucleic acid amplification. DNA was extracted with the QIAamp DNA Investigator Kit (Qiagen). A duplex quantitative real-time PCR (2qPCR) targeting the ribosomal RNA gene internal transcribed spacer (ITS2) of *T. canis* and *T. cati* was performed as described by Durant et al. (2012). HCMV CSF DNA viral load was screened with the GeneProof Cytomegalovirus (CMV) PCR Kit (GeneProof). EBV DNA viral load was detected and quantified using the EBV R-gene kit (Biomérieux). *Toxoplasma gondii* was detected by real-time PCR with the *Toxoplasma g.* ELITe MGB® Kit (ELITech Group). DNA of herpesviruses HSV-1 and 2, VZV, HHV-6, HHV-7, and HHV-8 was screened using the VIASURE Herpes Virus 1, Herpes Virus 2 & Varicella Zoster Virus and Human Herpes Virus 6, 7 & 8 real-time PCR Detection Kits (Certest Biotec) according to the manufacturer's instructions. All experiments were blinded and performed in duplicate.

Statistical analysis

The analyses were performed with the R statistical software version 4.3.0 (R Development Core Team, 2023). Descriptive analyses were conducted to summarize patient and group characteristics. Normality was assessed by QQ plots and the Shapiro–Wilk test. Quantitative data were expressed as means and standard deviations (SDs), while qualitative data were presented as absolute counts and percentages. In the univariate analysis, significance was assessed using the χ^2 test or Fisher's exact test for categorical variables and the Student's t-test or Mann–Whitney tests for continuous variables when appropriate. A two-tailed p-value <0.05 was considered statistically significant. Univariate analyses were used for descriptive purposes.

In order to determine the independent risk factors for AD, a multivariable logistic regression analysis was performed. Prior to model fitting, multicollinearity among predictor variables was assessed using the Variance Inflation Factor (VIF). This was particularly important due to the potential correlation between age and seropositivity to various microorganisms, as older individuals were expected to have both a higher likelihood of AD and increased rates of seropositivity. Variables with a VIF greater than 5 were excluded to reduce redundancy and improve model stability. Additionally, to assess potential confounding by age on pathogen seropositivity, we performed a series of independent logistic regressions with age as the sole predictor for each serological marker, with adjustment for multiple comparisons using both Bonferroni and Benjamini – Hochberg (FDR) corrections. The final selection of variables was based on prior scientific evidence, biological plausibility, and VIF values below 5. Odds ratios (OR) with their 95% confidence interval (CI95%), and p-values were calculated for each variable. The significance of the model was evaluated by the p-value of the log-likelihood ratio (p(LRT)). A final simplified model including significant variables was performed. Precision-recall and receiver operating characteristic (ROC) curves were represented. A similar model was applied when comparing rapidly progressive cases of AD and non-rapidly progressive cases of AD.

The model performance was evaluated by calculating the accuracy and the area under the ROC curve (AUC). Specificity, sensitivity, false positive rate, and false negative rates were calculated. Additionally, it was internally validated by the bootstrap technique, as this method offers more accurate estimates of predictive accuracy than split-sample or k-fold cross-validation in small datasets. The logistic regression model was fitted for reference. Five hundred bootstrap iterations

were generated to fit the model and calculate the optimism-corrected AUC. Iterations with invalid values (NA) were excluded. The mean AUC and its confidence interval were calculated using percentiles.

Results

Study population

Out of 721 eligible patients, 79 were included in the study: 44 cases (11 rapidly progressing, 33 non-rapidly progressing) and 35 controls (Figure 1). Due to strict inclusion criteria, age and sex matching was not feasible, as all eligible controls were included. Controls were significantly younger than cases (63 vs. 71 years, $p < 0.001$), with no differences in sex distribution. Habitat, socioeconomic status, and comorbidities were similar, except for cerebrovascular disease and chronic ischemic heart disease, which were more frequent in AD patients (Table 1). A multivariable adjustment was applied to account for these differences in the analysis. AD biomarkers showed higher levels of T-tau, P-tau181, NfL, and lower Aβ1–42 levels and Aβ1–42/Aβ1–40 ratios in AD patients, who also had lower scores on neuropsychological tests (Table 2). The ApoE E4 allele was more frequent in AD ($p < 0.001$). Further information is available in Tables 1 and 2.

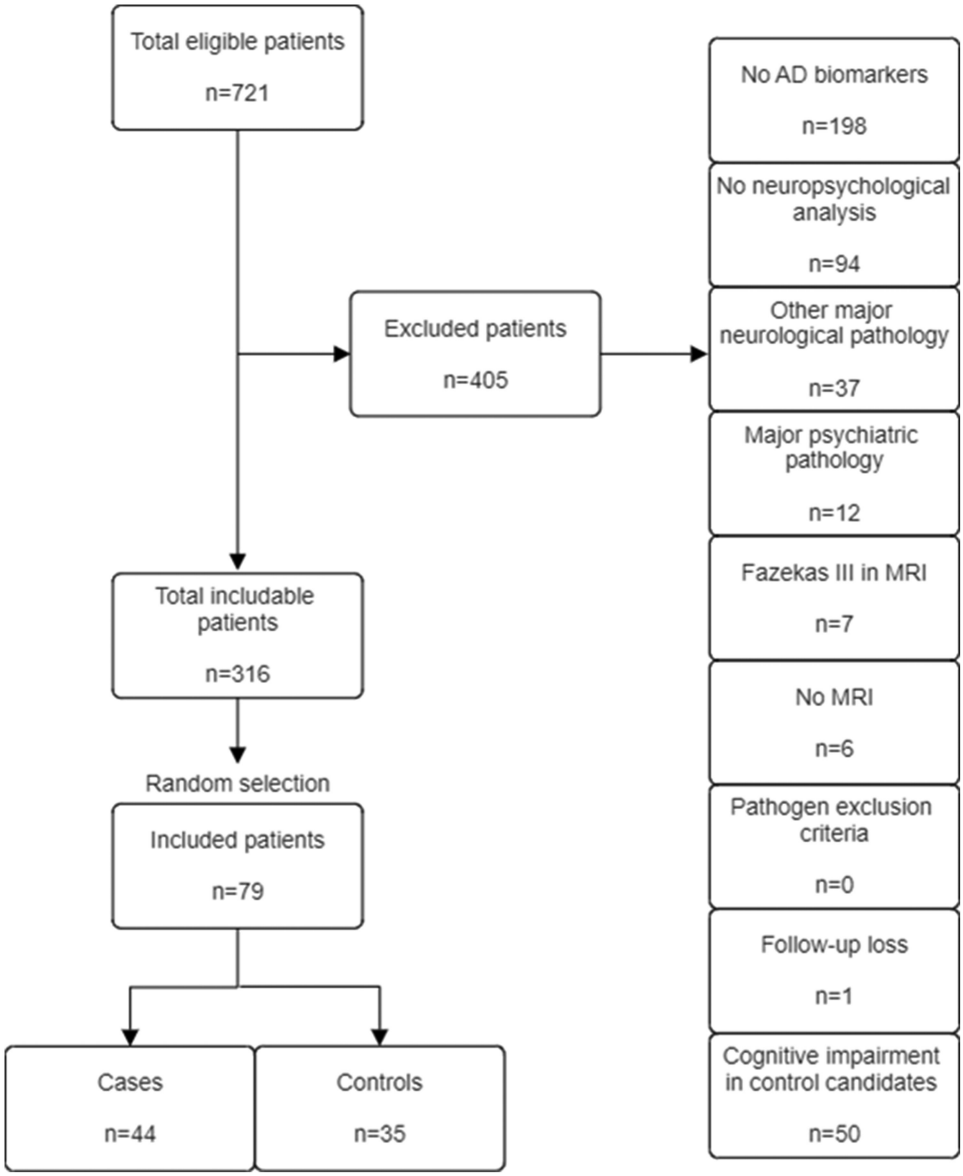

Figure 1. Flowchart of patient selection and exclusion criteria.
AD: Alzheimer's disease; MRI: magnetic resonance imaging.

Table 1. Demographic and clinical characteristics of patients with Alzheimer's disease (AD) and healthy controls.

Variable		AD patients Mean (SD) – n (%)	Healthy controls Mean (SD) – n (%)	p-value
Age (years)		70.88 (3.92)	63.46 (7.83)	Mann-Whitney $p < 0.001$
Sex	Men	20 (45.45)	17 (48.57)	Fisher $p = 0.823$
	Women	24 (54.55)	18 (51.43)	
Habitat	Urban	37 (84.09)	30 (85.71)	Fisher $p = 1$
	Rural	7 (15.91)	5 (14.29)	
Socioeconomic status	High	7 (15.91)	4 (11.43)	χ^2 $p = 0.273$
	Middle	32 (72.73)	30 (85.71)	
	Low	5 (11.36)	1 (2.86)	
Study level	Primary or basic education	29 (65.91)	20 (57.14)	χ^2 $p = 0.319$
	Secondary education	2 (4.55)	6 (17.14)	
	Intermediate vocational training or high school diploma	3 (6.82)	3 (9.57)	
	Higher vocational training or university degree	10 (22.73)	6 (17.14)	
Tobacco use		13 (29.55)	13 (37.14)	Fisher $p = 0.63$
Alcohol		12 (27.27)	8 (22.86)	Fisher $p = 0.477$
Hypertension		17 (38.64)	13 (37.14)	Fisher $p = 1$
Type 2 diabetes mellitus		6 (13.64)	8 (22.86)	Fisher $p = 0.377$
Dyslipidemia		26 (59.09)	20 (57.14)	Fisher $p = 1$
Mild cerebrovascular disease		4 (11.43)	0 (0)	Fisher $p = 0.035$
Chronic ischemic heart disease		5 (14.29)	0 (0)	Fisher $p = 0.014$
Autoimmunity		1 (2.27)	4 (11.43)	Fisher $p = 0.165$
Chronic kidney disease		1 (2.27)	1 (2.86)	Fisher $p = 1$
Statins use		22 (50)	20 (57.14)	Fisher $p = 0.651$
Antihypertensives use		17 (38.64)	13 (37.14)	Fisher $p = 1$
Antiepileptics use		0 (0)	0 (0)	NA
Antidepressants use		16 (36.36)	17 (48.57)	Fisher $p = 0.359$
NSAIDs use		6 (13.64)	6 (17.14)	Fisher $p = 0.757$
Corticosteroids use		1 (2.27)	0 (0)	Fisher $p = 1$
Immunosuppressants use		0 (0)	1 (2.86)	Fisher $p = 0.443$

NSAID: non-steroidal anti-inflammatory drugs.

Table 2. Biomarker profiles and cognitive assessment in patients with Alzheimer's disease (AD) and healthy controls.

Variable		AD patients Mean (SD) – n (%)	Healthy controls Mean (SD) – n (%)	p-value
Aβ1–42 (pg/mL)		623.18 (184)	1174 (465.66)	< 0.001
T-tau (pg/mL)		697.93 (311.90)	280.91 (140.24)	< 0.001
P-tau181 (pg/mL)		115.52 (61.75)	37.51 (18.32)	< 0.001
Aβ1–40 (pg/mL)		12772.7 (4094.65)	11649.8 (4238.09)	0.24
Aβ1–42/Aβ1–40		0.050 (0.01)	0.107 (0.03)	< 0.001
T-tau/Aβ1–42		1.206 (0.73)	0.516 (1.69)	< 0.001
Neurofilament light (pg/mL)		1266.36 (549.26)	740.45 (508.91)	< 0.001
Mini-mental state examination		21.73 (5.23)	27.37 (2.49)	< 0.001
CDR scale		0.75 (0.412)	0.40 (0.26)	< 0.001
CDR total points		4 (2.92)	1.74 (1.81)	< 0.001
CDR Memory		1.02 (0.59)	0.50 (0.34)	< 0.001
CDR Orientation		0.77 (0.56)	0.27 (0.33)	< 0.001
CDR Judgment and problem solving		0.99 (0.74)	0.43 (0.51)	< 0.001
CDR Community affairs		0.49 (0.51)	0.26 (0.41)	0.021
CDR Home and hobbies		0.52 (0.44)	0.23 (0.31)	< 0.001
CDR Personal care		0.23 (0.13)	0.06 (0.24)	0.123
RBANS Immediate memory		63.15 (16.47)	82.20 (16.02)	< 0.001
RBANS Visuospatial		76.15 (20.07)	89.94 (18.41)	0.001
RBANS Language		72.17 (16.43)	84.37 (15.65)	0.002
RBANS Attention		71.56 (18.91)	80.14 (18.11)	T test $p < 0.001$
RBANS Delayed memory		53.02 (15.46)	86.29 (15.57)	< 0.001
RBANS Total sum		337.60 (64.93)	423.371 (66.11)	T test $p < 0.001$
RBANS Battery total scale		60.23 (13.92)	81.03 (15.55)	< 0.001
RBANS Classification	Average/Mild impairment (≥70)	9 (20.5)	NA	NA
	Moderate impairment (55–69)	11 (25)		
	Severe impairment (< 54)	24 (54.4)		
ApoE genotype (alleles)	E2/E2	0 (0)	1 (2.86)	χ^2 $p = 0.001$
	E2/E3	0 (0)	3 (8.57)	
	E2/E4	1 (2.27)	0 (0)	
	E3/E3	15 (34.09)	22 (62.86)	
	E3/E4	24 (54.55)	9 (25.71)	
	E4/E4	3 (6.82)	0	
Fazekas scale	0	17 (38.64)	22 (62.86)	χ^2 $p = 0.06$
	1	17 (38.64)	11.31 (43)	
	2	9 (20.45)	2 (5.71)	

CDR: Clinical Dementia Rating.

RBANS: Repeatable Battery for the Assessment of Neuropsychological Status.

The type of statistical test is indicated only for variables with normal distribution (Student's t-test). For all other quantitative variables, the Mann-Whitney U test was used by default due to lack of normality.

Pathogen seroprevalence and molecular detection in CSF

A univariate analysis was performed to assess the differential seroprevalence between groups (Table 3). HMCV seropositivity was significantly more prevalent in AD patients (95.45%) compared to non-Alzheimer's (77.14%) ($p = 0.019$). Furthermore, a significantly higher seroprevalence of *C. burnetii* was observed in AD patients (22.73%) compared to controls (2.86%) ($p = 0.019$) (Table 3).

A sub-analysis of seroprevalence rates for all tested pathogens was performed based on rural versus urban living habitats. Both show high seroprevalence rates for HCMV, EBV, HSV, and VZV, while *C. burnetii* and *M. pneumoniae* had lower exposure rates independent of the residing location. For most pathogens, including *T. gondii* and *C. burnetii*, there were no significant differences in seroprevalence between rural and urban habitats. A non-significant trend toward higher positivity in urban habitats (71.64%) compared to rural (41.67%) was observed for *C. pneumoniae* ($p = 0.053$).

There were no significant differences in the prevalence of *Toxocara* spp., herpesviruses, *C. pneumoniae*, *M. pneumoniae*, and *T. gondii* between both groups. Only one positive result for *Toxocara* spp. IgG was observed, which occurred in a control patient. Varicella zoster virus and *T. gondii* IgG quantification was neither significantly different (Table 3).

Serological tests and PCR for all studied pathogens including HHV-6, HHV-7, and HHV-8 were also performed on CSF samples, with no positive results.

Further analyses were performed within the AD group, comparing rapidly progressive AD cases ($n = 11$) and non-rapidly progressive AD cases, as seen in Table 4. No statistically significant differences were seen in the univariate analysis of seroprevalences or antibody titers between both groups.

Multivariable regression analysis

A first multivariable regression model was developed incorporating age, ApoE genotype, cerebrovascular disease, autoimmunity, chronic ischemic heart disease, and seropositivity for *Toxocara* spp., HCMV, EBV, HSV, VZV, *C. pneumoniae*, and *C. burnetii* to explore potential independent predictors associated to AD in this population. Before model fitting, we assessed potential confounding by age on pathogen seropositivity both through VIF analysis and a series of independent logistic regressions with age as the sole predictor for each serological marker. After adjusting for multiple comparisons using both Bonferroni and FDR corrections, only HCMV seropositivity remained significantly associated with age (adjusted $p < 0.05$), whereas all other markers showed no significant association (Supplementary Table S1). These results, along with low collinearity indices (VIF < 5), and the inclusion of age in the multivariable model assured its robustness and indicate that residual confounding by age is unlikely to meaningfully affect the main associations observed.

The final model selected age (OR 1.55, CI95% 1.17–2.03, $p = 0.002$), HCMV seropositivity (OR 20.02, CI95% 1.03–-388.32, $p = 0.047$), and *C. pneumoniae* seropositivity (OR 8.60, CI95% 0.96–77.25, $p = 0.054$) as potential independent predictors of AD group in our cohort (p(LRT) <0.001). However, these estimates – particularly for HCMV and *C. pneumoniae*—were imprecise, with wide confidence intervals reflecting uncertainty in the strength of association due to small sample size. These variables were included in a simplified model which was then assessed and internally validated using the bootstrap technique in 500 replicated datasets. The accuracy of the simplified model was 74.68% (CI95% 0.6364–0.838), with an AUC of 0.807% (Figure 2). The sensitivity was 65.71% and specificity was 81.82%, with a positive predictive value of 74.19%.

After internal validation in 500 bootstrap samples, the adjusted logit model AUC was 0.792 (CI 95% 0.755–0.808).

Table 3. Seroprevalence and IgG levels of various pathogens in patients with Alzheimer's disease (AD) vs healthy controls.

Variable	AD patients	Healthy controls	p-value
Toxocara spp. n (%)	0 (0)	1 (2.86)	0.443
Human cytomegalovirus n (%)	42 (95.45)	27 (77.14)	0.019
Epstein-Barr virus n (%)	42 (95.45)	35 (100)	0.50
Herpes simplex virus n (%)	42 (95.45)	31 (88.57)	0.398
Varicella zoster virus n (%)	42 (95.45)	31 (88.57)	0.398
Chlamydia pneumoniae n (%)	31 (70.45)	22 (62.86)	0.63
Coxiella burnetii n (%)	10 (22.73)	1 (2.86)	0.019
Mycoplasma pneumoniae n (%)	6 (13.64)	6 (17.14)	0.757
Toxoplasma gondii n (%)	27 (61.36)	18 (51.43)	0.493
IgG quantification n (%)			
Varicella zoster virus IgG levels (mIU/mL) Mean (SD)	1048.55 (667.29)	985.85 (652.26)	0.409
Toxoplasma gondii IgG levels (IU/mL) Mean (SD)	35.68 (49.36)	24.60 (31.86)	0.689

Fisher test was used for qualitative variables and Student t test was used for quantitative variables.

Table 4. Differential seroprevalence and IgG levels of pathogens in rapidly vs. non-rapidly progressive Alzheimer's patients.

Variable	Rapidly progressive Alzheimer ($n = 11$)	Non-rapidly progressive Alzheimer ($n = 33$)	p-value
Human cytomegalovirus (n (%))	11 (100)	31 (93.94)	1
Epstein-Barr virus (n (%))	10 (90.91)	32 (96.97)	0.442
Herpes simplex virus (n (%))	11 (100)	31 (93.94)	1
Varicella zoster virus (n (%))	11 (100)	31 (93.94)	1
Chlamydia pneumoniae (n (%))	8 (72.73)	23 (69.70)	1
Coxiella burnetii (n (%))	4 (36.36)	6 (18.18)	0.237
Mycoplasma pneumoniae (n (%))	2 (18.18)	4 (12.12)	0.63
Toxoplasma gondii (n (%))	6 (54.55)	19 (57.58)	1
IgG quantification (n (%))			
Varicella zoster virus IgG levels (mIU/mL) Mean (SD)	982.09 (490.90)	1070.70 (721.81)	0.708
Toxoplasma gondii IgG levels (IU/mL) Mean (SD)	18.82 (37.53)	41.39 (52)	0.059

Fisher test was used for qualitative variables and Mann Whitney U test was used for quantitative variables.

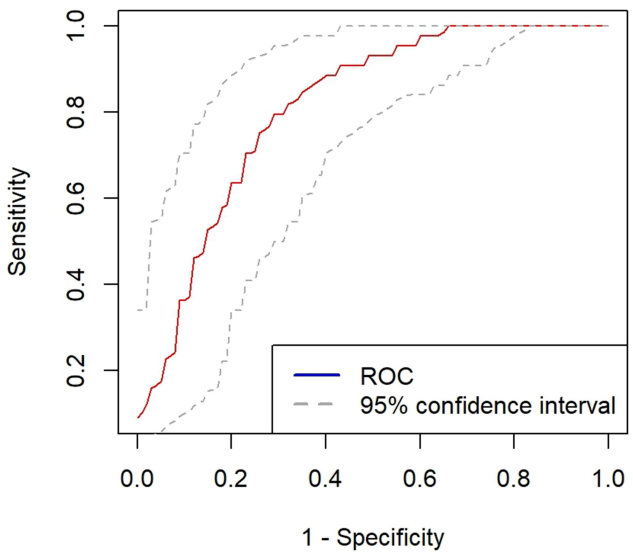

Figure 2. Receiver operating characteristic (ROC) for the multi-pathogen predictive model performance in Alzheimer's disease.

A similar multivariable approach was performed to compare rapidly progressive AD patients and non-rapidly progressive AD cases. Due to the lower sample size, only previously significant variables were included. Age, seropositivity to *C. pneumoniae*, HCMV, and *C. burnetii* were included in the model. Age (OR 1.42, CI95% 1.05–1.91, $p = 0.02$) and seropositivity to *C. burnetii* (OR 3.47, CI95% 1.32–778.06, $p = 0.03$) were associated with rapid progression, although the estimate for *C. burnetii* was also imprecise.

Details on "triple positive" patients

Three AD patients had combined seropositivity to HCMV, *C. pneumoniae*, and *C. burnetii* ("triple positive"). All of them were rapidly progressive AD patients, compared to 18.6% of observed rapid progression in those with no triple positivity ($p = 0.01$). No significant differences were observed in their age. This group also showed a lower Aβ40/Aβ42 ratio (0.05 vs 0.1, $p = 0.04$), higher NfL levels (1707.7 pg/mL vs 819.7 pg/mL, $p = 0.04$) and poorer cognitive performance (MMSE 18.67 vs 25.46, $p = 0.04$; RBANS 48 vs 76.88, $p = 0.03$). However, given the very small number of triple-positive individuals ($n = 3$), these results must be considered descriptive and hypothesis-generating, and should be interpreted with caution.

Discussion

In this case–control study, we explored the multivariable relationship between AD and infectious agents potentially implicated in its onset or progression using serological (IgG levels) and molecular microbiologic markers in both serum and CSF under strict inclusion criteria. Seropositivity for HCMV and *C. burnetii* was more frequent among AD patients, with *C. burnetii* also associated with a more rapidly progressive clinical course. After multivariable adjustment, age, HCMV seropositivity, and *C. pneumoniae* seropositivity seemed to be independent predictors of AD with good accuracy and internal validity. While confidence intervals for HCMV and *C. pneumoniae* were wide – reflecting some uncertainty in the estimated effect sizes – the overall pattern supports a potential contribution of these pathogens to AD. Exploratory findings in "triple positive" patients (seropositive for HCMV, *C. pneumoniae*, and *C. burnetii*) showed more rapid disease progression and worse cognitive performance and CSF impaired biomarker profiles compared to those without this triple positivity.

With recent advances in understanding neuroinflammation and immune dysregulation in AD pathophysiology, infection has garnered attention due to its association with similar pathological immune processes involved in AD development and neurodegeneration [14,15]. Several pathogens have been implicated in the onset and progression of AD and our findings go in parallel with previous works from both basic and clinical approaches analyzing the potential role of pathogens such as *C. pneumoniae* and HCMV, among others. Despite various animal models, clinical and necropsy evidence remains scarce. Our study and others suggest

that *C. pneumoniae* is a leading candidate for infectious involvement in AD. Despite its prevalence in AD brains [16] and previous animal models indicating common pathways to AD, definitive studies on its causative or contributory role in AD pathology and cognitive decline are lacking [17]. Additionally, there is insufficient serological evidence of chronic or past infections in AD patients and controls.

C. pneumoniae may influence brain biology and contribute to AD through systemic interactions, Trojan horse mechanisms, and direct nasal infection [17]. Systemic cytokine production from lung infections can cross into the brain, while elementary bodies can spread via the circulatory system. As an intracellular pathogen, *C. pneumoniae* resides in immune cells that can migrate to the brain [18]. Direct nasal infection has shown that *C. pneumoniae* can infect both glial and neuronal cells [19]. Microglial cells potentially support a persistent infection [20], which may contribute to AD by increasing senile plaques, neurofibrillary tangles, and neuroinflammation [16]. This pathogen has been shown to elevate amyloid-beta (Aβ) production in animal models and in vitro [19,21–23] and correlate with lower CSF Aβ42 and higher CSF tau [24], which may at the end be related to increased neurodegeneration and cognitive impairment.

Although clinical information on the relationship between *C. pneumoniae* and AD is limited, serological evidence suggests a potential link between HCMV chronic infection and AD, aligning with our results. A cohort study found that HCMV seropositivity was associated with an increased risk of AD and faster cognitive decline in older adults [25]. Another work reported associations between HCMV antibody levels and neurofibrillary tangles, as well as increased senescent T cells in HCMV-seropositive subjects [26]. These findings are consistent with recent evidence showing that HCMV seropositivity is associated with plasma biomarker evidence of Aβ accumulation [27], and murine models suggesting that MCMV infection induces AD-associated alterations in tau and neurodegeneration [28]. However, a meta-analysis of seven observational studies revealed heterogeneous results, with stronger associations observed in East Asian populations, cohort studies, and studies with confounder adjustments [29].

While our results concur with previous clinical and experimental results, our findings relating to *C. burnetii* are more uncertain and require further research, as it has not yet been an extensive object of study [30]. This intracellular pathogen with known neurotropic potential in rare cases can establish chronic latent infection and induce sustained systemic inflammation, endothelial dysfunction, and immune dysregulation and potential molecular mimicry – all mechanisms increasingly implicated in AD pathogenesis [31]. Despite its theoretical plausibility as a contributor to neurodegeneration that led us to include it in the serological model, and isolated mentions in the literature hypothesizing its involvement in AD [30], direct studies on its involvement in AD are scarce. This gap highlights the need for targeted research to validate our exploratory results, limited by strong imprecision, in larger cohorts or in pathological analyses, and clarify whether *C. burnetii* may act as a peripheral inflammatory driver or co-factor in the complex, multifactorial etiology of AD.

In this work, we used a well-defined unicentric case–control design with strict inclusion criteria and random patient selection, detailed patient characterization, and advanced serological and molecular analyses. Robust statistical methods, including multivariable logistic regression and bootstrap validation to adjust to potential confounders such as age, were employed to contribute to understanding infectious involvement in AD. Despite no causal evidence can be extracted from case–control studies, our multiparametric approach aligns with the current trends in the microbial pathogenesis hypothesis of AD, suggesting that AD is not associated with a single pathobiont. Instead, it highlights the potential role of multi-pathogen infections and the coexistence of a complex interplay of multiple pathogens in the etiology of AD [9].

Notably, despite detecting seropositivity for various pathogens in serum, all CSF PCR analyses returned negative results. This divergence highlights key questions about the mechanisms through which pathogens may influence AD. The absence of microbial DNA in CSF samples suggests that active neuroinfection by these pathogens was unlikely at the time of sampling. Several interpretations are possible: first, the findings may reflect systemic or peripheral effects of chronic or latent infections rather than direct CNS invasion – consistent with emerging evidence that systemic inflammation and immune activation can affect the brain in neurodegenerative diseases. Second, certain pathogens may reside in brain tissue or cells without shedding detectable nucleic acids into the CSF which would be more indicative of active severe infection, particularly in latent or low-replication states. Third, technical limitations may have contributed, including the limited sensitivity of PCR to detect low-copy DNA in CSF, especially in the absence of acute infection. Finally, these pathogens may have been truly absent from the CNS in our cohort. While our serological findings point toward prior systemic exposure, the lack of CSF detection supports the notion that any

contribution to AD pathogenesis may be indirect, mediated through peripheral immune mechanisms, molecular mimicry, or by priming neuroinflammatory cascades rather than via direct infection of the central nervous system [11,12,17,32].

Non-significant associations for other pathogens may reflect high background seroprevalence with limited sample size and statistical power, or confounding by unmeasured factors such as overall health or lifetime inflammation. Reverse causality is also possible given the study's cross-sectional design.

However, several important limitations deserve mention. The low sample size, resulting from stringent criteria to minimize bias and a unicentric and retrospective nature, limits the generalizability and statistical power of the results. In particular, the observations related to the triple-positive subgroup are primarily descriptive and should be interpreted as hypothesis-generating, given the extremely limited number of individuals in this category, and should be validated in larger cohorts. Matching cases and controls by age and sex was not feasible, as no more available controls were available due to the strict inclusion criteria to eliminate bias and the difficulty of having CSF samples from healthy population. We acknowledge that HCMV seropositivity increases with age and could potentially act as a surrogate marker of immunosenescence. To account for this, we included age as a covariate in the multivariable logistic regression model, and additionally confirmed that multicollinearity between age and pathogen seropositivities was minimal (all VIF values <5). Furthermore, we conducted independent logistic regressions to evaluate whether age significantly predicted seropositivity for each pathogen. Except for HCMV, no significant associations were observed after multiple testing correction, suggesting that residual confounding by age is unlikely to explain the associations found. Importantly, HCMV remained an independent predictor of AD even after adjusting for age, supporting a potential biological role consistent with previous studies linking chronic HCMV infection with cognitive decline, neuroinflammation, and AD-related pathology. Additionally, PCR analysis for *C. pneumoniae* and *C. burnetii* in CSF was not performed due to the absence of validated protocols and reliable positive controls for these targets in our laboratory at the time of the study, as well as their non-inclusion in routine workflows. Moreover, evidence for direct CNS invasion by these pathogens was limited at the time of study design. We acknowledge this as a limitation, as it prevents direct assessment of neuroinvasion, though our serological approach remains relevant for exploring systemic infectious contributions to AD. Finally, while the internal validation is robust, it does not substitute for external validation in other cohorts, which is necessary for broader result extrapolation.

Conclusion

These findings suggest that latent infections with HCMV, *C. pneumoniae*, and *C. burnetii* may play a role in AD pathophysiology, possibly through systemic or immune-mediated mechanisms. While the associations observed are consistent with prior hypotheses, they should be interpreted in light of certain limitations, including the modest sample size, lack of age matching, and statistical imprecision. Further research in larger, well-characterized cohorts is importantly needed to replicate these findings and better define the potential contribution of infectious burden to AD.

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Disclosure statement

No potential conflict of interest was reported by the author(s).

Data availability statement

The data that support the findings of this study are openly available in Figshare at https://doi.org/10.6084/m9.figshare.29365508.v1.

Ethics approval and consent to participate

The study protocol was approved by the Ethics Committee of the Health Research Institute La Fe with registration code 2023/181–1 and was compliant with the Declaration of Helsinki. All patients gave informed consent for the use of their biological samples.

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