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RESEARCH ARTICLE

C1q is increased in cerebrospinal fluid-derived extracellular vesicles in Alzheimer's disease: A multi-cohort proteomics and immuno-assay validation study

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Abstract

Introduction: Extracellular vesicles (EVs) may propagate and modulate Alzheimer's disease (AD) pathology. We aimed to comprehensively characterize the proteome of cerebrospinal fluid (CSF) EVs to identify proteins and pathways altered in AD.

Methods: CSF EVs were isolated by ultracentrifugation (Cohort 1) or Vn96 peptide (Cohort 2) from non-neurodegenerative controls ($n = 15, 16$) and AD patients ($n = 22, 20$, respectively). EVs were subjected to untargeted quantitative mass spectrometry-based proteomics. Results were validated by enzyme-linked immunosorbent assay (ELISA) in Cohorts 3 and 4, consisting of controls ($n = 16$, $n = 43$, (Cohort3, Cohort4)), and patients with AD ($n = 24$, $n = 100$).

Olaf Jahn and Anja Schneider contributed equally to this work.

Results: We found > 30 differentially expressed proteins in AD CSF EVs involved in immune-regulation. Increase of C1q levels in AD compared to non-demented controls was validated by ELISA (~ 1.5 fold, p (Cohort 3) = 0.03, p (Cohort 4) = 0.005).

Discussion: EVs may be utilized as a potential biomarker and may play a so far unprecedented role in immune-regulation in AD.

KEYWORDS
Alzheimer's disease (AD), biomarker, cerebrospinal fluid (CSF), complement, extracellular vesicles, immune system, mild cognitive impairment (MCI), proteomics

1 | INTRODUCTION

Extracellular vesicles (EVs) are vesicles of 50-200 nm diameter that are released by all CNS cells, including neurons,[1] astrocytes,[2] oligodendrocytes,[3] and microglia.[4] EVs play important roles in cell-cell communication and release of toxic cell content, thereby modulating critical cellular processes such as signaling, metastasis,[5] angiogenesis,[6] or inflammation.[7] EVs have been implicated to contribute to spreading of disease pathology in many neurodegenerative diseases including Parkinson's disease (PD),[8] amyotrophic lateral sclerosis,[9] and Alzheimer's disease (AD).[10]

AD is characterized by predominantly extracellular deposition of aggregated amyloid-β (Aβ) in the brain, and intracellular accumulation of hyperphosphorylated tau in neurofibrillary tangles.[11] The pathology of many neurodegenerative diseases starts to develop in a particular area of the brain and then slowly spreads to other regions in a specific pattern.[12] However, how these aggregated proteins are propagated from one area of the brain to another is still enigmatic. Recently, many studies have suggested transfer of Aβ and tau from one cell to another via EVs as one of the possible mechanisms.[13] It has been shown that EVs derived from AD patients' brains carry higher levels of Aβ oligomers and may act as mediators for the inter-neuronal transfer of these oligomers to recipient cultured neurons.[14] Transneuronal transfer by EVs was also reported for phosphorylated tau[15] and AD biomarkers Aβ42, total tau (tTau) and phosphorylated tau (pTau) have been shown to be differentially expressed in CSF and plasma EVs derived from AD patients.[16] EVs may be double-edged swords. On one hand, they may be involved in clearance of pathological proteins from the cell, and on the other hand, this mechanism could propagate these proteins throughout the brain.

Over the past decade, evidence has accumulated that inflammation plays a central role in AD, exacerbating both Aβ and tau pathology.[17] Interestingly, EVs have been shown to modulate and/or propagate inflammatory processes, for example, the NLRP3 inflammasome pathway[18] and mechanistic links between EVs and innate immunity have been investigated.[19] One such innate immune pathway is the complement system that may contribute to EV-mediated pro- and anti-inflammatory immune balance as well as synaptic loss.[20] Complement proteins associated with EVs have been shown to be elevated in diseases that possess both neuroinflammatory and neurodegenerative components such as multiple sclerosis[20] and AD and traumatic brain injury.[21]

The main aim of this study was to identify novel CSF EV-associated biomarkers. To get a global view of the EV associated proteins or pathways that may be altered in AD and providing novel insights into the role of EVs in neurodegeneration there is a need to identify CSF EV protein profiles in neurodegenerative diseases. Moreover, EV proteins may serve as diagnostic and predictive biomarkers and aid in the stratification of patients for clinical trials. CSF derived EVs can closely reflect the underlying pathologies in the central nervous system (CNS) as CSF is a direct window to the brain. It has been shown previously that CSF EVs contain proteins of CNS origin.[22] We aimed to investigate (i) the CSF EV protein profiles of AD and non-neurodegenerative controls (Cohort 1 and 2) using an unbiased proteomics approach and (ii) validated the most promising candidate C1q along with two other promising biomarkers aldolase C (ALDOC) and cathepsin B (CTSB) in two other cohorts comprising patients with AD, mild cognitive impairment (MCI) and non-demented controls (Cohorts 3 and 4) using a commercially available enzyme-linked immunosorbent assay (ELISA). Altogether, we show that EVs from CSF are a powerful tool for biomarker development.

2 | METHODS

2.1 | Human CSF samples

The overall study design is shown in Figure 1. We used two independent cohorts, recruited in Göttingen and Amsterdam: Cohorts 1 (Göttingen) and 2 (Amsterdam) as discovery cohorts for proteomics, Cohort 3 and 4 comprised of additional and independent samples of the Göttingen and Amsterdam cohort, as validation cohorts for ELISA measurements.

2.1.1 | Cohort 1 and Cohort 3

Human CSF samples were collected from non-demented, non-neurodegenerative controls (n = 15) and patients with Alzheimer's disease dementia (n = 22) (Cohort 1) at the Department of Psychiatry and Psychotherapy at University Medical Center Göttingen

(Germany). The samples were obtained with the approval of the ethical committee of the Medical Faculty, University Medicine Goettingen (IRB 02/05/09) in accordance with the ethical standards outlined in the 1964 Declaration of Helsinki. All AD dementia patients fulfilled the National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA) and National Institute on Aging and Alzheimer's Association (NIA-AA) diagnosis criteria for probable AD dementia.[23]

Following the same procedures, and additionally with CSF biomarker data, 16 non-demented controls, 24 patients with AD were included in Cohort 3 which was collected independently from Cohort 1 at the Department of Psychiatry at University Medical Center Göttingen (Germany) (IRB 02/05/09). CSF Aβ42, total tau (tTau), and phosphorylated tau (pTau181) were measured as a part of routine diagnosis using commercially available ELISA kits according to the manufacturer's protocol (Innogenetics NV, Ghent, Belgium) with cutoffs for Aβ42 < 450 pg/ml, tTau > 200 pg/ml, and pTau181 > 61 pg/ml. The diagnosis of AD pathology was based on positive CSF Aβ42 and tTau levels, whereas all controls were negative for these markers. Control CSFs included CSF from cognitively healthy patients with depression, cephalgy, schizophrenia, bipolar disorder, and polyneuropathy or were obtained from a cohort of healthy subjects that had undergone neuropsychological testing to rule out cognitive impairment. All CSF samples were collected by lumbar puncture according to previously published JPND-BIOMARKAPD guidelines.[24,25] In short, ~10 ml of CSF was obtained between 9 and 12 am in polypropylene CSF collection tubes, centrifuged at 2000 × *g* for 10 min at room temperature (RT), aliquoted, and frozen at −80 °C within 30 min of completion of the procedure.

Demographic and clinical details of all patients are listed in Table 1.

2.1.2 | Cohort 2 and Cohort 4

Human CSF samples were collected from the Amsterdam Dementia Cohort (ADC)[26] for Cohort 2, which comprised of 16 non-demented controls and 20 AD patients. Cohort 4 was independently collected from ADC comprising 43 non-demented controls and 100 AD patients. Diagnoses were defined in a multidisciplinary committee according to the criteria of the NINCDS-ADRDA.[23] Diagnosis of AD patients and selection of non-demented controls were as published previously.[27] CSF Aβ42, tTau, and phosphorylated tau (pTau181) were measured as a part of routine diagnosis using commercially available ELISA kits according to the manufacturer's protocol (Innogenetics NV, Ghent, Belgium) with cutoffs for Aβ42 < 813 pg/ml, tTau > 470 pg/ml and pTau181 > 52 pg/ml. Samples within each cohort were matched for age. Demographic and clinical details of all patients are listed in Table 1.

2.2 | Neuropsychological procedures

All subjects were examined with standardized neuropsychological tests as described previously.[27] The Consortium to Establish a Registry for Alzheimer's Disease (CERAD) neuropsychological test battery includes verbal and visuospatial learning subtests with immediate and delayed recall, a naming test, a verbal fluency test, and the Mini-Mental State Examination test (MMSE).

RESEARCH IN CONTEXT

1. **Systematic Review**: The literature was reviewed by the authors using online resources such as PubMed, Web of Science, and Scopus. While extracellular vesicles (EVs) may play important roles in Alzheimer's disease (AD) pathology, there is no multi-cohort study yet on CSF EVs proteome combining different EV isolation techniques. Recent publications related to immune-modulatory proteins associated with AD CSF-derived EVs are cited.
2. **Interpretation**: Several immune system related proteins including complement protein C1q, derived from CSF EVs, are increased in AD. EVs may play a so far unprecedented role in immune-regulation and inflammation in AD and may be utilized as potential biomarkers in MCI and AD.
3. **Future Directions**: Future studies should investigate how the CSF EV proteome is altered during the course from preclinical to manifest AD stages using larger longitudinal cohorts, and whether the impact of cellular C1q release with EVs fulfills a protective function or merely reflects increased C1q expression in AD.

2.3 | Extracellular vesicle (EV) isolation from CSF

2.3.1 | Ultracentrifugation

For all cohorts except for Cohort 2, ultracentrifugation was used for CSF EV isolation. The starting volume of CSF was 0.5 ml for all cohorts except for Cohort 4, where 1 ml was used. CSF samples were thawn on ice and then subjected to serial centrifugation at 4°C and 3500 × *g*, two times at 4500 × *g* (10 min each) and one time at 4°C and 10,000 × *g* for 30 min using low-bind Eppendorf tubes (Cat. Number 0030122283). Next, the supernatant was transferred to an ultracentrifugation tube (Beckman, Cat. Number: 344088) and centrifuged at 100,000 × *g* for 1 h at 4°C followed by washing the pellet with phosphate buffered saline (PBS) and repeating the ultracentrifugation step. EVs were characterized by Western blotting (WB), nanoparticle tracking analysis (NTA), and transmission electron microscopy (TEM) (supplemental methods and Figure S1) according to MISEV guidelines.[28]

2.3.2 | Affinity capture by Vn96 peptide

EV isolation using Vn96 peptide[29,30] was performed for CSF from Cohort 2 samples only. 0.5 ml of CSF was incubated with 25 μg of

TABLE 1 Demographic and clinical details of all patients.

Proteomics	Cohort 1 Controls	Cohort 1 AD	Cohort 2 Controls	Cohort 2 AD	ELISA	Cohort 3 Controls	Cohort 3 MCI-AD	Cohort 3 AD	Cohort 4 Controls	Cohort 4 MCI-AD	Cohort 4 AD
N	15	22	16	20	n	16	12	12	43	50	50
Sex	F = 10, M = 5	F = 12, M = 10	F = 7, M = 9	F = 8, M = 12	Sex	F = 8, M = 8	F = 7 M = 5	F = 3, M = 9	F = 17, M = 26	F = 22, M = 28	F = 21, M = 29
Age (years (mean ± SD)	64 ± 7	69 ± 7[a]	61 ± 7	63 ± 6	Age (years (mean ± SD)	60 ± 11	70 ± 11	72 ± 9[a]	63 ± 6	68 ± 6[c]	68 ± 7[a]
MMSE[§] (mean ± SD)	28 ± 2	23 ± 3[a]	28 ± 1	19 ± 6[a]	MMSE[§] (mean ± SD)	28 ± 3	26 ± 3[c]	24 ± 4[a]	26 ± 3	26 ± 2	23 ± 5[a]
tTau (pg/ml) (median[IQR])	175 [150–203]	382 [259–1162][a]	217 [174–240]	658 [455–1066][a]	tTau (pg/ml) (median[IQR])	148 [122–168]	474 [303–624][c]	449 [240–773][a]	223 [185–283]	635 [505–806][c]	663 [536–860][a]
pTau181 (pg/ml) (median[IQR])	41 [32–46]	85 [51–172][a]	35 [32–41]	82 [52–111][a]	pTau181 (pg/ml) (median[IQR])	38 [29–41]	88 [59–107][c]	86 [47–110][a]	42 [31–47]	90 [73–104][c]	93 [75–105][a]
$A\beta_{42}$ (pg/ml) (median[IQR])	1221 [851–1333]	671 [569–1009][a]	1010 [953–1132]	598 [520–721][a]	$A\beta_{42}$ (pg/ml) (median[IQR])	1059 [936–1352]	686 [581–888][c]	647 [572–860][a]	1050 [971–1194]	642 [541–716][c]	647 [512–805][a]
					C1q (pg/ml) (median[IQR])	2794 [2224-5688]	6938 [3547-21352]	6402 [3511-13143][a]	29670 [15395-55250]	6938 [3547-21352][c]	46680 [26650-77780][a]
					Annexin (pg/ml) (median[IQR])	1833 [1313-2473]	1815 [1786-2456]	1749 [1398-2351]	447 [258-652]	1.9 [1.7-2.4]	336 [207-639]
					C1q/Annexin	1.92 [1.201-2.664]	2.76 [1.841-7.367]	2.81 [1.841-7.367][a]	74.5 [45.8-137.9]	123.5 [57.65-258.6]	120.2 [62.76-243.6][b]

[§] MMSE, Mini-Mental State Examination.

[a] $p < 0.05$, [b] $p < 0.01$ AD versus controls

[.c] $p < 0.05$ MCI versus controls

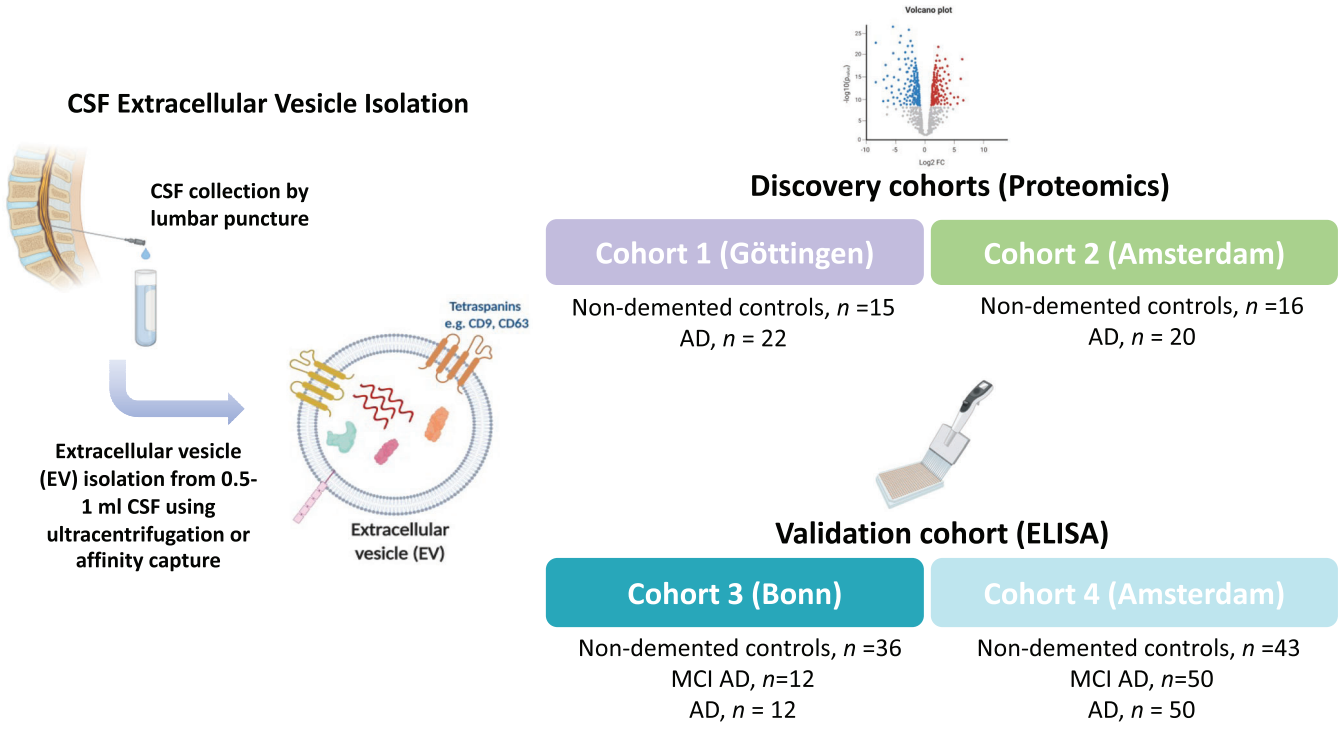

FIGURE 1 Schematic showing the study design. Cohort 1 (Göttingen), Cohort 2 (Amsterdam) were used for proteomics analysis. Cohort 3 (Bonn) and Cohort 4 (Amsterdam) were used for ELISA analysis. This figure was created using BioRender.com.

Vn96 peptide and protease inhibitor cocktail (Cat. Number 10276200, Roche) for 1 h at RT. The samples were then centrifuged at 16, 000 × *g* for 10 min at RT. The EV pellet was stored at −20°C until further analysis. EVs were characterized according to MISEV guidelines using WB and NTA (Figure S2).[28]

2.4 | Label-free protein quantification Cohort 1

Sample preparation, data acquisition, and data analysis were essentially performed as previously described in detail for synaptic protein fractions from mouse brain. EVs in the ultracentrifugation pellet were dissolved in 200 μl lysis buffer (2% CHAPS, 7 M urea, 2 M thiourea, 10 mM DTT, 0.1 M Tris pH 8.5) and subjected to automated in-solution digestion by filter-aided sample preparation (FASP). Tryptic peptides were separated by nanoscale reversed-phase UPLC separation and mass spectrometric analysis was performed in ion mobility-enhanced data-independent acquisition (DIA) mode. ISOQuant was used for post-identification analysis and calculation of absolute in-sample amounts for each detected protein according to the TOP3 method. For additional details, please refer to the Supporting Information.

2.5 | Label-free protein quantification Cohort 2

Proteomics has been performed as described previously.

2.6 | Analysis of proteome data

To improve comparability of the discovery datasets, an identical database was used for searching the mass spectrometric data from Cohort 1 (proteome analysis performed in Göttingen) and Cohort 2 (proteome analysis performed in Amsterdam), respectively. For additional details, please refer to the Supporting Information.

2.7 | ELISA

CSF EV preparations were lysed with 1% CHAPS in PBS and stored at −80°C until further analysis. CSF EV samples were thawed on ice and C1q and Annexin A1 levels were measured by commercially available ELISA (C1q: Cat. Number SEA747Hu, Cloud-Clone Corp., Katy, TX, USA; Annexin A1: Cat. Number MBS704042, MyBioSource, San Diego, CA, USA (Cohort 3 was measured with the kit before 2020, Cohort 4 with the kit after 2020. Please note that the calibrator of this kit was changed in 2020); fructose-bisphosphate aldolase C (ALDOC): Cat. no. ABIN6007462, Genomics, Aachen, Germany; Human Total Cathepsin B: Cat. no. DY2176, Bio-Techne, Minnesota, USA). Annexin A1 values were used for C1q data normalization to account for the possible differences in EV particle numbers in different patient samples. CSF Aβ42, tTau, and pTau181 were measured as a part of routine diagnosis with commercially available ELISA kits according to the manufacturer's protocol (Innogenetics NV, Ghent, Belgium).

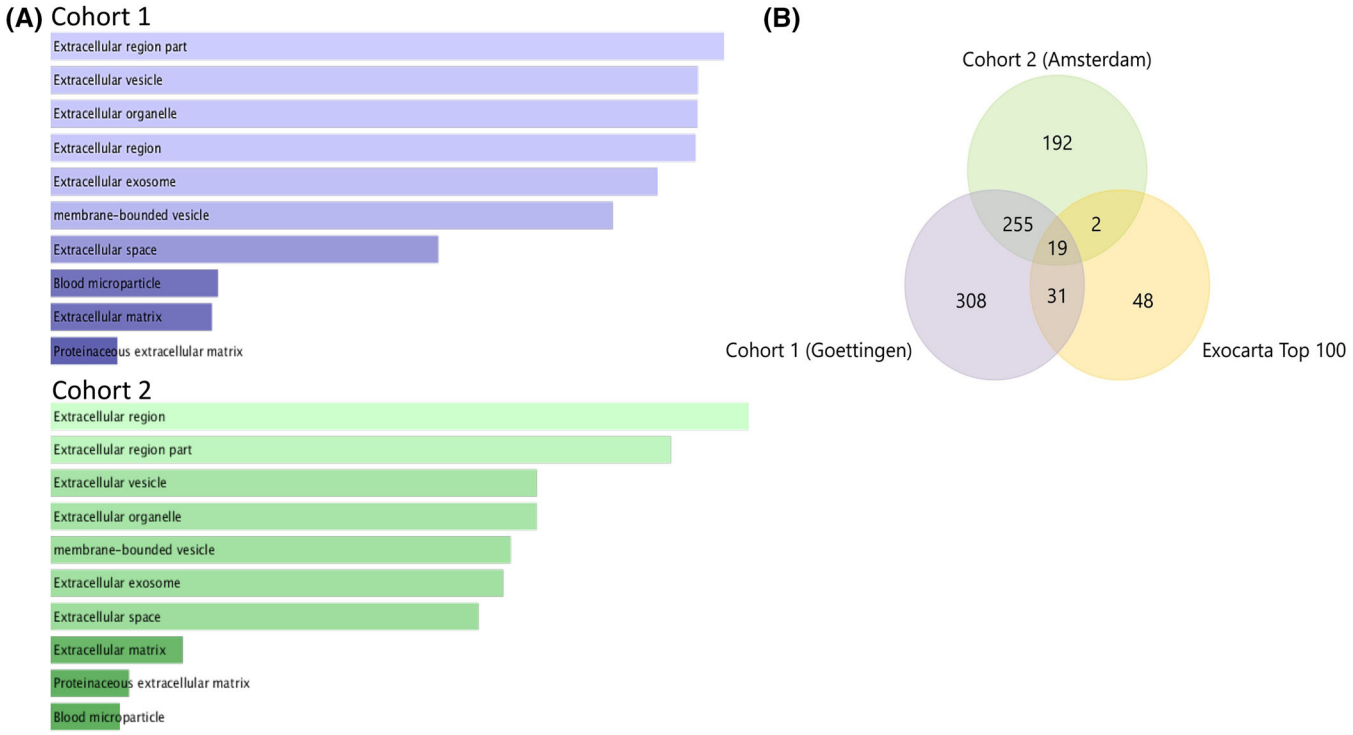

FIGURE 2 (A) All identified proteins in Cohort 1 (lilac) and Cohort 2 (green) enriched for cellular compartment localization led to identification of terms related to extracellular vesicles/exosomes. (B) Overlap of all proteins in Cohort 1 and Cohort 2 with ExoCarta top 100 proteins (yellow).

2.8 | Statistical analysis

Data were measured while blinded to the diagnosis. Biological pathway enrichment was performed using Enrichr. Differences in C1q concentrations measured by ELISA were tested with Mann-Whitney and Kruskal-Wallis tests for non-parametric data. Correlation analyses were performed using Spearman correlation. The statistical tests were two-tailed and values with $p < 0.05$ were considered significant. Analysis of covariance (ANCOVA) and statistical analyses were performed on SPSS version 22 (IBM SPSS Statistics for Windows, Version 21.0; IBM Corp., Armonk, NY, USA). Graphs were plotted using GraphPad Prism version 7.05.

3 | RESULTS

3.1 | Proteomic profiling of CSF EVs

3.1.1 | Cohort 1 and Cohort 2

CSF EVs were prepared by ultracentrifugation from Cohort 1 and subjected to label-free quantification (LFQ) of proteins using in-solution digestion followed by ion mobility-enhanced data-independent acquisition (DIA) mass spectrometry. In total, we detected 613 proteins, of which 50 have been identified in ExoCarta top 100 proteins (exocarta.org) (Figure 2), including EV marker such as CD9, CD63, and PDCD6IP (ALIX). ExoCarta is an online database platform that catalogs exosome specific data pertaining to proteins, RNAs and lipids (http://www.exocarta.org).[31] It is a primary resource of exosomal cargo[32] and contains annotations on the isolation and characterization methods. We tested the quality of EVs isolated using each technique- ultracentrifugation and Vn96 by analyzing their proteome overlap with ExoCarta as it is one of the biggest databases for EV-associated proteins (data accumulated from several hundred published papers on EV proteins). The top 100 proteins in ExoCarta are the most frequently detected ones in EVs.

In Cohort 2, EVs were affinity-captured from CSF using the synthetic Vn96 peptide which binds to canonical heat shock proteins localized on the EV surface. In contrast to ultracentrifugation, Vn96 purification is better suitable for routine diagnostics as it does not require specialized laboratory equipment and allows EV preparation in a short time,[33] however on the expense of EV populations that are devoid of HSP surface proteins. EVs were and subjected to LFQ of proteins using in-gel digestion followed by data-dependent acquisition (DDA) mass spectrometry, we identified 468 proteins, of which 274 proteins overlapped with Cohort 1 and 21 proteins overlapped with ExoCarta top 100 proteins. The lists of all proteins identified in these cohorts are provided in Table S1 and S2. Enrichment of all identified proteins for cellular compartment localization revealed terms such as extracellular vesicle, membrane bound vesicle, or similar in both Cohorts 1 and 2 (Figure 2). This indicates that both methods are effective in isolating EVs form CSF, yet, show slight differences in

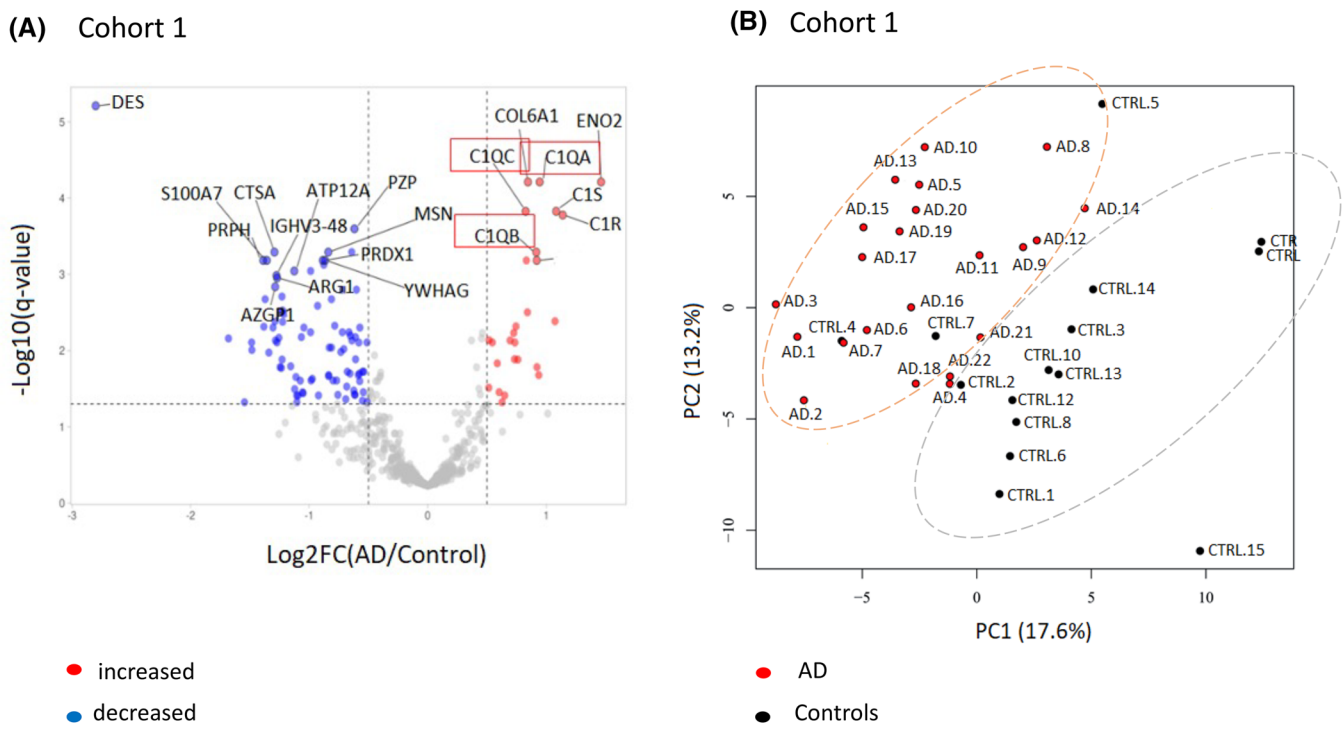

FIGURE 3 (A) Volcano plot showing AD versus non-demented controls comparison in Cohort 1. C1q was found to be increased as highlighted within red boxes. Fold change (log2FC) cutoff = 0.5, Significance (-Log10) = 1.3 (B) PCA (Principal component analysis) representing clear proteomics profile differences between AD patients (red solid circles) and controls (black solid circles).

protein composition and number of identified proteins. Since affinity purification may represent only a subset of the total EV population present in CSF, we decided to perform all validation steps using ultracentrifugation purified EVs.

Analysis of the protein abundance data from Cohort 1 by Pearson correlation (Figure S3) and principal component analysis (PCA, Figure 3A) revealed some separation of the two conditions, indicating differences in the proteome profiles between AD patients and controls. Indeed, 114 proteins were significantly altered (*q.mod*, i.e., *p*-value corrected for false discovery rate (FDR) < 0.05) in CSF EV samples of AD patients versus controls with 26 proteins significantly increased and 88 proteins significantly decreased with fold change ($\log_2$FC) cutoff = 0.5 and significance (-$\log_{10}$ q-value) cut-off = 1.3 (Figure 3B). Notably, CSF EV-associated complement proteins C1q, C1r, and C1s were all increased in AD compared to non-demented controls (Figure 3B). Gene ontology (GO) biological pathway enrichment (overrepresentation analysis) of increased proteins revealed regulation of complement activation, regulation of immune effector process, and regulation of humoral response as highly represented pathways (Figure 4). The decreased proteins were linked among others to neutrophil degranulation and neutrophil activation (Figure 4).

In Cohort 2, though there was 59% overlap of identified proteins with Cohort 1, only three proteins were found to be up-regulated applying the same cutoffs for fold change and significance. Similar to Cohort1, C1q was up-regulated in Cohort 2, but the *q.mod* value was not significant (Figure S4).

3.2 | Validation of C1q and additional biomarkers using ELISA

3.2.1 | Cohort 3

We next validated complement factor C1q, which was up-regulated in AD versus controls, using a commercially available ELISA. CSF EV C1q levels were validated, since the complement system has been implicated in AD pathology. Moreover, in animal models of AD, the opsonization of synapses by C1q was shown to contribute to neurodegeneration,[34] and in human AD brains, C1q is highly expressed and associated with amyloid plaques.[35] In Cohort 3, CSF EV C1q levels were significantly higher in the AD group, compared to controls ($p = 0.03$) (Figure 5A) with an area under the curve (AUC) of 0.7840 (Figure S8).

When the AD group was stratified into MCI-AD and mild AD dementia, we found that C1q levels tended to be higher in MCI due to AD ($p = 0.07$) compared to controls (Figure 5B). Overall, higher levels of C1q in AD are in line with our proteomics data from Cohort 1, where this protein was also found to be increased in CSF EVs.

3.2.2 | Cohort 4

Since C1q was found to be significantly increased in Cohort 3, we validated this biomarker in a larger cohort, Cohort 4. In Cohort 4, similar to

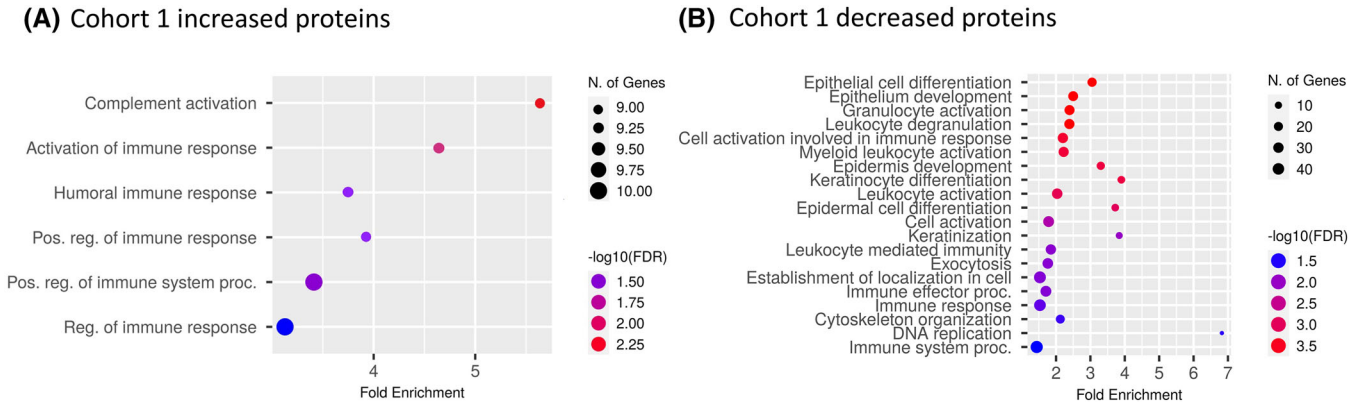

FIGURE 4 26 increased (A) and 89 decreased proteins (B) in AD were enriched for gene ontology (GO) biological process. Complement system and Innate immune system related processes were enriched in increased and decreased proteins respectively.

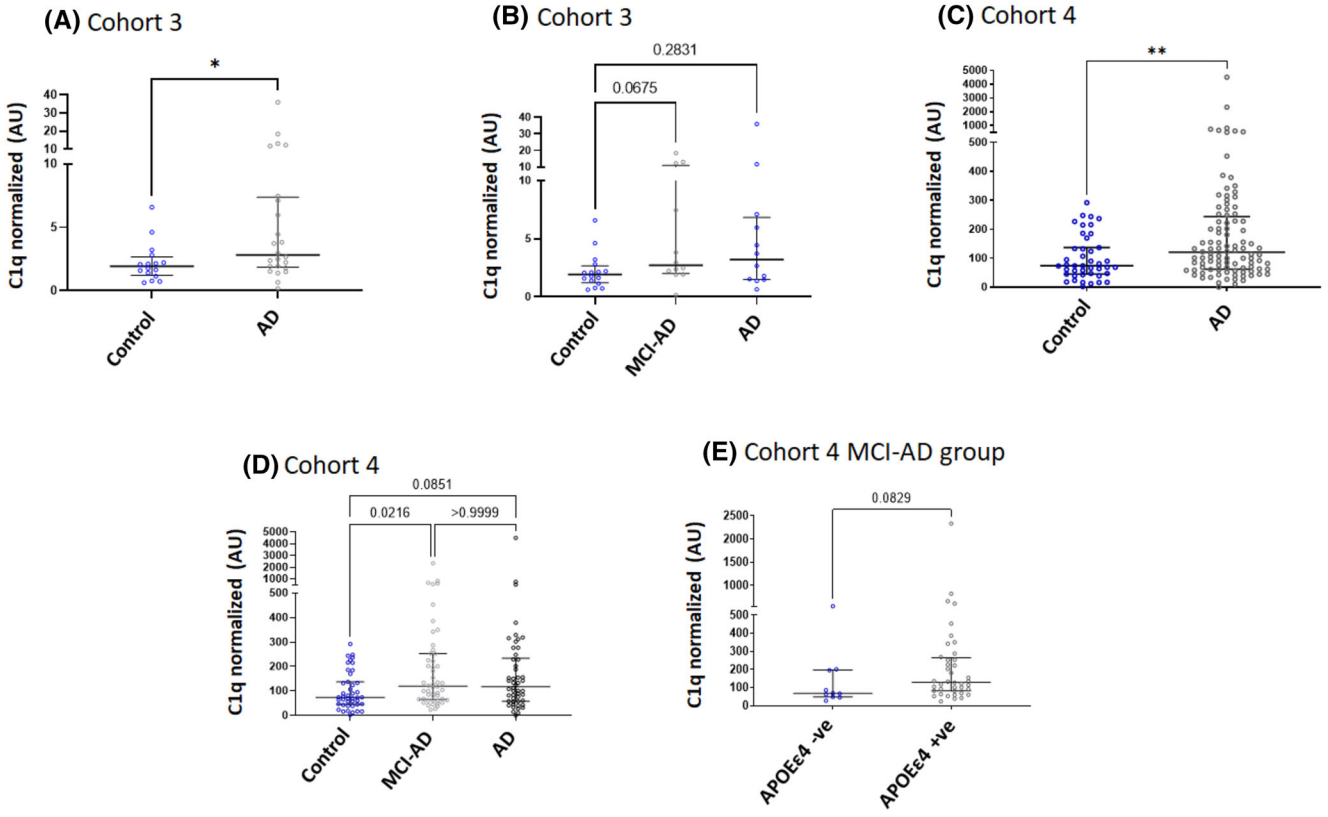

FIGURE 5 (A) Levels of CSF EV associated C1q in Cohort 3 (Control and combined AD group). (B) Cohort 3 combined AD group was stratified into MCI-AD and AD dementia. CSF EV associated C1q. (**C**) Levels of CSF EV associated C1q in Cohort 4 (Control and combined AD group). (**D**) Cohort 4 combined AD group was stratified into MCI-AD and AD dementia. CSF EV associated C1q. (**E**) The MCI-AD of Cohort 4 was stratified by *APOE*ε4 carrier status (negative versus positive). CSF EV associated C1q. Mann-Whitney U test was performed for two-group comparison (A, C, and E). Kruskal-Wallis test with Dunn's correction for multiple comparison was performed (B and D). The long horizontal lines represent median and short horizontal lines represent inter-quartile range. $^{*}p < 0.05$. $^{**}p < 0.01$.

Cohort 2, CSF EV C1q levels were significantly higher in the AD group compared to controls ($p = 0.005$) (Figure 5C) with an AUC of 0.8280 (Figure S8).

When the AD group was further stratified, EV C1q levels were higher in MCI due to AD ($p = 0.02$) compared to controls. However, similar to Cohort 3, the elevation of EV C1q levels in AD dementia only group could not reach statistical significance (Figure 5D). Interestingly, there was a tendency ($p = 0.08$) of higher C1q levels in *APOE*ε4+ve (carrying at least one *APOE*ε4 allele) compared to *APOE*ε4-ve (carrying no *APOE*ε4 allele) within the MCI-AD group (Figure 5E).

3.2.3 | Validation of proteins decreased in AD CSF EVs

Additionally, we validated two CSF EV associated proteins that were found to be decreased in our proteomics dataset from Cohort 1, aldolase C (ALDOC) and cathepsin B (CTSB). Both ALDOC and CTSB have been previously implicated in AD pathology. ALDOC (fructose-bisphosphate aldolase C) is a glycolytic protein that among other functions has been reported in neutrophil degranulation, and was shown to be decreased in AD brain entorhinal cortex.[36] CTSB is a lysosomal cysteine protease that can play a role in neutrophil degranulation[37] was described as a driver of microglial inflammation,[38] and in the production of neurotoxic amyloid species as well as in its degradation.[39] We found that in both the cohorts, CSF EV associated ALDOC ($p < 0.0001$) and CTSB ($p < 0.0001$) were significantly decreased in AD compared to controls (Figure S5, Table S3), further supporting our proteomics data. In addition, we measured C1q, ALDOC, and CTSB levels in neat CSF. Whereas C1q levels in neat CSF were higher in the AD group, ALDOC and CSTSB CSF levels did not differ between AD and controls (Figure S6).

3.2.4 | Correlation analysis for CSF EV C1q

Correlation analysis revealed a significant correlation of EV C1q levels and MMSE in both, MCI and AD groups ($r = 0.32$, $p = 0.02$; $r = 0.29$, $p = 0.04$, Figure S7), whereas CSF C1q did not correlate with MMSE scores in these diagnostic groups ($r = -0.074$, $p = 0.756$; $r = -0.130$, $p = 0.547$, Figure S7). We additionally performed correlation analysis of EV C1q to CSF markers of Aβ42, tTau, and pTau in the larger of the two cohorts, that is, Cohort 4, separately for MCI-AD ($n = 50$), AD dementia ($n = 50$), and the combined AD group ($n = 100$). We found a significant negative correlation between EV C1q and tTau in the MCI group ($r = -0.317$, $p < 0.0314$), whereas all other correlation analyses did not reach significance (Figure S9).

No correlation was found between CSF EV C1q levels and age or sex ($r = 0.0892$, $p = 0.0652$). ANCOVA analysis to control CSF EV C1q levels for age and sex status did not change the results (Cohort 3 ($p = 0.03$: age and sex corrected p-value = 0.039), (Cohort 4 ($p = 0.005$: age and sex corrected p value = 0.0056), (Cohort 4 AD group (*APOE*ε4- ve & *APOE*ε4+ ve) ($p = 0.0829$; age and sex corrected p value = 0.0864).

4 | DISCUSSION

EVs are a rich source of biomarkers, yet under-explored in CSF using unbiased proteomics. They have been implicated in the pathogenesis of AD as putative sites for processing of amyloid precursor protein (APP)[40] as well as mediators of disease propagation, for example, by intercellular transfer of aggregated tau.[4,15,41] However, the role of EVs in AD remains poorly understood and only very few studies explored EVs derived from AD patients or from CSF using proteomics.[22,42,43]

To our knowledge, this is the first study on AD CSF EVs that included four independent cohorts from different centers, the comparison of two CSF EV isolation methods, ultracentrifugation and affinity purification, the use of different unbiased proteomics approaches, and finally the validation of candidate biomarkers by ELISA. We identified C1q as a potential CSF EV associated biomarker that was increased in MCI due to AD, and in a combined MCI and AD group compared to non-neurodegenerative disease controls.

Among the important issues to be considered in biomarker studies is that the applied technique is feasible for clinical applications, accessible, and fast.[44] Multiple techniques applied for EV isolation have been described such as ultracentrifugation and affinity capture by the Vn96 peptide. Here, we did not perform additional isolation to separate small EVs after UC. However, due to the sequential centrifugation with $10.000 \times g$ centrifugation prior to ultracentrifugation, we obtain EVs in a size range of 50-250 nm as measured using the nanoparticle tracking analyzer. Since we did not separate the EV preparation further, we use the general term 'extracellular vesicle (EV)' and not exosome or small EVs, according to the MISEV guidelines.[45]

UC is the most commonly used gold standard method for EV isolation, whereas the Vn96-peptide-based method is easier, faster, requires smaller sample volumes, and can be performed with standard laboratory equipment.[46,47] However, it only captures the EV subpopulation which carries HSP on its surface. Importantly, although we used two different techniques for EV isolation (UC vs. Vn96-peptide-based affinity purification) and two different proteomic approaches for the quantification of CSF EV proteins (in-solution tryptic digestion/DIA mass spectrometry vs. in-gel tryptic digestion/DDA mass spectrometry), we found that similar biological pathways changed in AD compared to non-demented control groups.

Our proteomics results from Cohort 1 showed 613 proteins quantified in CSF EVs, with 26 increased and 88 proteins decreased in AD compared to controls (Table S1). The majority of the decreased proteins were linked to neutrophil mediated immunity, whereas the majority of increased proteins in AD CSF EVs represented immune-related pathways, most prominently the complement cascade such as C1q, C1s, C1r, C4, and CFH. Other increased proteins related to the innate immune system were A2M (alpha-2-macroglobulin) and CD93.

As these findings indicated that CSF may be enriched in EVs derived primarily from microglia, we compared our CSF EV protein list from Cohort 1 with a proteome resource of mouse brain cell types separated by magnetic-activated cell sorting (MACS) and analyzed by the same proteomic approach as used here.[48] This resource preferentially covers the cell body proteomes of **a**strocytes, **o**ligodendrocytes, **n**eurons, **e**ndothelial cells, and **m**icroglia (here referred to as AONEM). Comparison of all 613 proteins quantified in CSF EV with the AONEM resource revealed that in principle all cell types contribute to the CSF EV pool, with some apparent prevalence of the glial cell types (Figure S10A). However, comparison of only the 114 CSF EV proteins with altered abundance in AD showed a predominant overlap with the microglial proteome (Figure S10B). We thus concluded that the inflammatory

protein signature observed here reflects a facet of AD pathology, rather than a general enrichment of microglial EVs in CSF.

Among the different proteins altered in CSF EVs in AD, we chose C1q for further validation. C1q binds to fibrillar Aβ and initiates the activation of the classical complement pathway.[49] Additionally, C1q has been linked to synapse loss, a major hallmark of AD, where C1q tags the synapses to be pruned by microglia even before plaque deposition starts,[50] indicating its involvement in early AD pathology. Genes coding for regulatory proteins of the complement cascade, complement receptor 1 (CR1), complement factor H and clusterin, were identified as risk genes for AD, further supporting an important role of the complement system in AD.[51] Increased EV-associated C1q could reflect a rescue mechanism of C1q tagged synapses to remove C1q by shedding of opsonized membranes to prevent pruning. A similar mechanism has been previously described for other cell types that shed the membrane attack complex which forms downstream of activated C1q via EVs. This notion is further supported by the positive correlation between MMSE and CSF EV C1q levels, indicating that patients with higher CSF EV C1q show a better cognitive performance. In addition, we found a negative correlation of CSF EV C1q with the neuronal injury marker tau in the MCI-AD group of Cohort 4, indicating that high levels of CSF EV C1q are correlated with less neuronal injury (Figure S9). Future studies are needed to answer the question of whether C1q release with EVs fulfills a protective function or merely reflects increased C1q expression in AD.

We further validated CSF EV C1q in two independent cohorts using a commercially available ELISA. In both cohorts, EV C1q was significantly higher in the combined AD group as well as in the MCI group compared to cognitively unimpaired controls. The increase of CSF EV C1q was more prominent in MCI (Figure 5B, D), consistent with findings by Hong et al.[50] who reported C1q opsonization of synapses to be an early event in the disease course. Our ELISA results further support that CSF EV associated C1q is a potential biomarker for early stages of AD and may reflect underlying neuro-inflammatory processes. It is interesting to note that C1q levels tended to be higher in *APOE*ε4+ve (carrying at least one *APOE*ε4 allele) compared to *APOE*ε4-ve (carrying no *APOE*ε4 allele) within in the MCI-AD group (Figure 5E). *APOE*ε4 is a genetic risk factor for sporadic AD and associated with plaque deposition, tau pathology[52] as well as neuroinflammation.[53] Higher CSF EV C1q levels could thus reflect the increased neuroinflammation observed in *APOE*ε4 carriers.

Next, the most prominent pathway that emerged from the proteomics analysis of decreased proteins in Cohort1 was related to neutrophil-mediated immunity. Neutrophil granule proteins may be involved in both neuroprotective and neurotoxic effects.[54] The role of neutrophils in AD is largely under-studied. However, several previous studies have reported an increase of neutrophil degranulation and activation in AD.[55] In contrast, based on our data from Cohort 1 CSF EVs, we found several neutrophil degranulation related proteins to be decreased in AD. This finding was validated by ELISA for ALDOC and CTSB in two independent cohorts.

The major strength of our study is its unbiased approach to identify dysregulated proteins in EVs derived from CSF of patients with AD and the validation of selected proteins by ELISA using two independent cohorts. CSF provides a direct window to the brain and EVs derived from CSF may closely reflect the underlying pathologies that may be propagated between CNS cells. Another advantage of our study is the isolation of CSF EVs from individual patients instead of using CSF pools or from very large CSF volumes which would not be feasible for biomarker detection in the routine practice. One limitation of our study is the small sample size of our cohorts. Despite this, we could replicate our findings from the proteomics AD cohorts in two independent cohorts using ELISA. Future studies should investigate how the CSF EV proteome is altered longitudinally in AD to uncover early molecular changes.

In conclusion, a unique inflammatory signature derived from circulating EVs of patients with AD could complement classical AD biomarkers and be implemented for patient stratification and monitoring treatment response in drug trials.

AUTHOR CONTRIBUTIONS

(1) Research Project: A. Conception, B. Organization, C. Execution; (2) Statistical Analysis: A. Design, B. Execution, C. Review and Critique; (3) Manuscript Preparation: A. Writing of the First Draft, B. Review and Critique.

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CONFLICT OF INTEREST STATEMENT

The authors report no competing interests relevant to this manuscript.

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Anja Schneider employed by German Center for Neurodegenerative Diseases, DZNE, Bonn, and University Hospital Bonn, has received honoraria for consultancy from Novartis, performs contract research for Eisai and Biogen and is on an advisory board for Biogen. Author disclosures are available in the supporting information.

CONSENT STATEMENT

Written informed consent was obtained from all participants before inclusion in the study. The medical ethics committee approved the study.

DATA AVAILABILITY STATEMENT

Proteomics datasets are available as supplementary Tables S1 and S2. The ELISA datasets analyzed in this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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