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Interrelations of Alzheimer´s disease candidate biomarkers neurogranin, fatty acid-binding protein 3 and ferritin to neurodegeneration and neuroinflammation

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Abstract

There is growing evidence that promising biomarkers of inflammation in Alzheimer´s disease (AD) and other neurodegenerative diseases correlate strongest to levels of tau or neurofilament, indicating an inflammatory response to neuronal damage or death. To test this hypothesis, we investigated three AD candidate markers (ferritin, fatty acid binding protein 3 (FABP-3), and neurogranin) in interrelation to established AD and inflammatory protein markers. We further aimed to determine if such interrelations would be evident in pathological subjects only or also under non-pathological circumstances. Cerebrospinal fluid levels of the three proteins were quantified in samples from the University Clinic of Bonn (UKB) Department of Neurodegenerative Diseases & Geriatric Psychiatry, Germany. Data were analyzed based on clinical or biomarker-defined stratification of subjects with adjustment for covariates age, sex, and *APOE* status. Levels of ferritin, FABP-3 and neurogranin were elevated in subjects with pathological levels of t-tau independent of beta-amyloid status. The three markers correlated with each other, tau isoforms, age, and those inflammatory markers previously described as related to neurodegeneration, predominantly sTREM2, macrophage migration inhibitory factor, soluble vascular endothelial growth factor receptor, soluble vascular cell adhesion molecule 1 (sVCAM-1), and C1q. These interrelations existed in subjects with pathological and sub-pathological tau levels, in particular for FABP-3 and neurogranin. Relations to ferritin were independent of absolute levels of tau, too, but showed differing trajectories between pathological and non-pathological subjects. A specific set of inflammatory markers is highly related to markers of neuronal damage such as tau, neurogranin, or FABP-3. These proteins

Abbreviations: AD, Alzheimer´s disease; C1q, complement factor C1q; CSF, cerebrospinal fluid; FABP, fatty acid binding protein; FCSRT, free and cued selective reminding test; MCI, Mild cognitive impairment; MIF, macrophage migration inhibitory factor; MMSE, mini mental state examination; ND, non-demented; NDs, neurodegenerative disorders; Nf-L, neurofilament light; PD, Parkinson´s disease; sICAM-1, soluble intercellular adhesion molecule 1; sTREM2, soluble triggering receptor expressed on myeloid cells 2; sVCAM-1, soluble vascular cell adhesion molecule 1; sVEGF-R, soluble vascular endothelial growth factor receptor.

could be used as readouts of the inflammatory response during the neurodegeneration phase of AD.

KEYWORDS

Alzheimer´s disease, biomarker, neurodegeneration, neuroinflammation

1 | INTRODUCTION

Neuroinflammation represent a characteristic feature of Alzheimer´s disease (AD) and other neurodegenerative disorders (NDs) (Edison & Brooks, 2018; Hampel et al., 2020; Heneka et al., 2015; Labzin et al., 2018). The time course of this inflammatory response likely spans over decades and is multi-facetted: First, pathogenic protein aggregates, such as beta-amyloid-, tau-, or synuclein-aggregates, stimulate microglial inflammasome activation and release of pro-inflammatory cytokines. Over several disease stages, the release of damage-associated molecular patterns (DAMPs) from dying neurons provides an additional inflammatory stimulus. The resulting chronic inflammation of the central nervous system (CNS) is detrimental and aggravates the disease phenotype and development. Currently, detectable inflammatory proteins in cerebrospinal fluid are most of all correlated with established markers of neuronal damage: Tau isoforms and neurofilament light (Nf-L) (Bettcher et al., 2018; Brosseron et al., 2018, 2019; Melah et al., 2016). Therefore, these proteins are likely to represent a set of inflammatory markers that reflect the CNS immune response to neuronal damage and could serve as a valuable readout for anti-inflammatory intervention studies at this stage of disease. If the concept is valid that neuronal death results in both the release of neuronal proteins and DAMPs that trigger specific inflammatory signals in reaction to the neuronal death, any cerebrospinal fluid (CSF) marker of neurodegeneration should correlate with these responsive inflammatory proteins CSF levels, just as observed for tau isoforms or Nf-L. To test this hypothesis, we quantified three candidate biomarkers of AD—neurogranin, fatty acid binding protein 3 (FABP-3) and ferritin—in cerebrospinal fluid (CSF) samples obtained from memory clinic outpatient unit patients at the University of Bonn Medical center.

Neurogranin is used as biomarker of dendritic and synaptic degeneration with high specificity for AD (Blennow & Zetterberg, 2018). Neurogranin is concentrated at dendritic spines, involved in synaptic signaling and within the brain only expressed by neurons (Díez-Guerra, 2010). Elevated neurogranin levels have been described in CSF of mild cognitive impairment (MCI) and AD patients. It correlates with levels of tau isoforms rather than with beta amyloid and is influenced by sex, age, and Apolipoprotein E (*APOE*) genotype (Kester et al., 2015; Kvartsberg, et al., 2015; Lista et al., 2017; Mattsson et al., 2016; Pereira et al., 2017; Portelius et al., 2015; Sanfilippo et al., 2016; Sun et al., 2016; Tarawneh et al., 2016; Thorsell et al., 2010; Wang & Alzheimer's Disease Neuroimaging Initiative, 2019; Wellington et al., 2016, 2018). Different neurogranin peptides can be detected by mass spectrometry, some of which are specific for plasma or CSF, respectively (Kvartsberg, et al., 2015). In contrast to CSF, plasma neurogranin levels seem not to differ between AD or controls (De Vos et al., 2015; Kvartsberg, et al., 2015; Palmqvist et al., 2019). Some studies have investigated neurogranin and YKL-40 as neurodegeneration/ neuroinflammation marker pair, but not further tested interactions between these two or with other inflammation markers (Hellwig et al., 2015; Höglund et al., 2017; Janelidze et al., 2016; Racine et al., 2019).

Fatty acid binding protein 3 (FABP-3 or heart-type FABP, H-FABP) is a small cytosolic protein with functions in fatty acid transport, metabolism, and energy demands and is primarily expressed in the heart, but also at lower levels by other organs including the brain (Thumser et al., 2014). FABP-3 has relevant functions in the adult brain, whereas the brain-type FABP (B-FABP or FABP-7) is more important for brain development. FABP-3 is expressed in acetylcholinergic and glutamatergic neurons and involved in neurite and synapse formation (Moullé et al., 2012). FABP-3 became of interest for the AD biomarker field because of different proteomic screenings and validation in subsequent targeted studies (Guo et al., 2013). Its increased CSF levels are interpreted as early markers of neuronal damage (Bjerke et al., 2011, 2016; Chiasserini et al., 2017; Harari et al., 2014; Höglund et al., 2017; Matsui et al., 2010; Olsson et al., 2013, 2016; Steinacker et al., 2004). Hence, it has been suggested to include FABP-3 in multi-modal biomarker models to characterize different NDs (Chiasserini et al., 2017; Llano et al. (ADNI), 2017; ; ; ; Lehallier et al., 2016). FABP-3 levels in CSF show good bio-stability over several months of sampling from the same subjects, further supporting its use as biomarker (Olsson et al., 2013; Trombetta et al., 2018).

The third protein assessed, ferritin, is an iron storage protein routinely used for iron deficiency testing that gained attention because of studies on iron homeostasis in AD and other NDs (Biasiotto et al., 2016; Cahill et al., 2009; Daru et al., 2017; Namaste et al., 2017; Nnah & Wessling-Resnick, 2018; Xu et al., 2017). Ferritin likely relates to AD in a multi-facetted manner throughout mechanisms of iron homeostasis that correlate with amyloid plaque load and neuronal loss including cellular senescence, γ-secretase activity, production and iron binding of amyloid beta (Aβ) and microglial iron accumulation (Avramovich-Tirosh et al., 2008; Bulk et al.., 2018; Kwiatek-Majkusiak et al., 2015; Li et al., 2013; Lopes et al., 2008; Masaldan et al., 2018; McCarthy et al., 2018; Pankhurst et al., 2008; Rogers et al., 2008, 2016; Ross, 2017; Thomsen et al., 2015; Venkataramani et al., 2018; Wang et al., 2017). Iron accumulation, in particular in the inferior temporal region, is furthermore correlated with cognitive decline, tau aggregation, and neurodegeneration in subjects with AD pathology (Ayton et al., 2019; Spotorno et al., 2020). Higher CSF and

plasma levels of ferritin are described as predictive of CSF Aβ levels, plaque load and reduced brain metabolism in individuals with pathological AD biomarker profile, and in particular in *APOE*-ε4 carriers (Ayton et al., 2018; Ayton et al., 2015; Ayton et al., 2017; Diouf et al., 2019; Goozee et al., 2018). Ferritin levels are furthermore influenced by demographic factors such as age or sex (Patton et al., 2017).

In this study, we tested how CSF levels of the above candidate markers relate to established AD hallmark markers—amyloid and tau—and how they correlate with markers of inflammation. For this purpose, we first verified if findings for the candidate markers in our cohort are in line with previous studies using bot clinical and amyloid/ t-tau based stratification schemes. Then, we analyzed in detail the interrelations of the candidate markers to inflammatory proteins considering potential covariates such as age in subjects with and without indicated neurodegeneration. Our results show that the three candidate markers are related to established neurodegeneration markers but independent of beta-amyloid pathology and are furthermore related to specific inflammation markers. These findings support the concept of an inflammatory biomarker profile that accompanies neurodegeneration in AD and other disorders and contains proteins that could serve as therapeutic targets or readout signals for anti-inflammatory intervention studies.

2 | METHODS

2.1 | Ethics approval and consent to participate

Informed consent for use of samples & data for research purposes was given with the local ethics committee approval (University Hospital of Bonn Ethics Commission #279/10). This work does not contain identifiable data of the subjects or any other specific individual person´s data.

2.2 | Study design

This study was not pre-registered. CSF levels of ferritin, FABP3, and neurogranin were determined in CSF samples and analyzed together with previously established data on clinical features as well as AD and inflammation markers (Brosseron et al., 2018). Statistical analysis first aimed to verify comparability of results to previous studies on the three markers by testing their relations to clinical diagnosis and AD biomarkers. For further clarification of their use as biomarkers of neurodegeneration, the candidate biomarker levels were analyzed considering blood–brain barrier (BBB) dysfunction and other disorders than AD. Subject numbers (N) for the different stratification methods used in this study are provided in Table 1. Next, the three markers were set in relation to markers of inflammation by principal component analysis (PCA) and a series of correlation analyses. These addressed which inflammatory proteins would be related to the three markers, how strong these

TABLE 1 Groups and stratification schemes

Stratification/Group	Whole Cohort (355)													
Disorder spectrum	AD spectrum (283)								Other disorders (72)					
Clinical (355)	ND (63)		MCI (100)			AD (120)			PD (21)	DLB (10)	FTD (21)	ALS (20)		
MCI subgroups (100)			MCI-A (39)	MCI-O (61)										
Clinical & BBB −/+ (235)	- (45)	+ (15)	- (74)	+ (15)		- (70)	+ (16)							
t-Tau positive	AD spectrum T− (133)				AD spectrum T+ (150)				Other disorders T− (57)		Other disorder T+ (15)			
A/T (355)	A−T− (84)		A−T+ (25)		A + T− (49)		A + T+ (125)		A−T− (40)	A−T+ (9)	A + T− (17)		A + T+ (6)	
A/T & BBB −/+ (235)	- (59)	+ (20)	- (16)	+ (5)	- (30)	+ (7)	- (84)	+ (14)						

Note: The table provides an overview of different stratification approaches used in this study for the cohort of subjects. Analysis was focused on the ND to AD spectrum of subjects, including amyloid positive MCI and other MCI cases. To supplement the AD spectrum, small groups of other disorders were included for comparison of trends. The cohort was also stratified based on routine biomarkers amyloid ratio and total tau (A/T scheme), likewise with focus on the ND to AD spectrum. For other diseases, these were tested as t-tau positive and by trend analysis using the A/T scheme. Covariates such as age, sex, and APOE status were included by ANCOVA analysis. Blood–brain barrier dysfunction data (no dysfunction, BBB-, versus. dysfunction BBB+) was available for a subset of the whole cohort and tested by sub-group analysis to clarify whether it would drive changes observed in CSF levels of the candidate markers ferritin, FABP-3 or, for example, because of potential transfer of proteins from peripheral blood.

Abbreviations: AD, Alzheimer´s disease; FABP, fatty acid binding protein; MCI, mild cognitive impairment; PD, Parkinson´s disease.

relations were and if they were dependent on neurodegeneration and/ or neuroinflammation. It was furthermore tested how relations of the markers to inflammatory proteins differ between subjects with or without neurodegeneration, and if elevated levels of the three markers would influence relations of immune markers to aging as critical risk factor of dementia.

2.3 | Samples and existing data

Samples and data of clinical features, amyloid- and tau biomarkers, as well as inflammation markers were derived from the biobank of the Department of Neurodegenerative Diseases & Geriatric Psychiatry, at the University of Bonn Medical Center, Germany. This biobank includes subjects by requesting participation from patients during their clinical diagnostic procedures, without active recruitment of specific patient groups and resulting in randomness of subjects from all groups of patients at the time of inclusion. After provision of informed consent, subjects' samples are assigned a sequential research ID that blinds laboratory personal to subject data or clinical group. Details on the setup of this biobank, procedures of sample collection, diagnostic criteria, beta-amyloid, and tau biomarker determination and the inflammation marker levels have been described previously (Brosseron et al., 2018). Blood–brain barrier (BBB) dysfunction was determined by measure of serum/CSF albumin quotient at the local central laboratory of the UKB, using age-dependent cut-offs for the albumin quotient. This study included all suitable samples of the biobank to maximize power without previous sample size calculation: A total of 355 samples comprised of 63 non-demented neurology patients (ND), 100 MCI, 120 AD, 21 Parkinson´s disease (PD), 21 frontotemporal dementia (FTD), 10 dementia with Lewy bodies (DLB), and 20 amyotrophic lateral sclerosis (ALS) patients. For stratification of patients by pathological AD biomarkers, the cut-offs were: Ratio Aβ42/40 <0.07; t-tau >450 pg/ml; p-tau-181 >56 pg/ml.

2.4 | Biomarker measurements

CSF levels of neurogranin, FABP3 and ferritin where determined using a commercially available bead-based immunoassay (MILLIPLEX MAP Human Neuroscience Magnetic Bead Panel 2, HNS2MAG-95K; Merck KGaA). In principle, the assay was performed following manufacturer's instructions. CSF dilution was adapted to 5x dilution to improve assay range of the target analytes. Washing steps were done using a handheld magnet (Handheld Magnetic Separator Block, 40-285; Merck KGaA). For readout, a MAGPIX® reader (Luminex Corporation) was used. Samples and calibrators were run in duplicates with a coefficient of variance (CV) <20%. An aliquoted internal control CSF sample was used to control for inter-run variances (<20%). Samples were measured in order of sample ID, resulting in arbitrary order of subjects from all tested groups, and laboratory personal was blinded to clinical diagnosis or other details of the samples by use of sequential IDs.

2.5 | Statistical analysis

Inflammation biomarker data used in this study were found to follow a skewed, non-normal distribution and statistical analysis as well as graphical visualization were performed as described elsewhere (Brosseron et al., 2018, 2019). In brief, Prism 8 (GraphPad Software Inc.) and IBM SPSS Statistics 21 (IBM Corporation) where used to calculate non-parametric group comparisons without exclusion of outliers (Kruskal–Wallis or Mann–Whitney *U* tests), receiver operating characteristics (ROC) and Spearman correlations. Groups were defined by clinical diagnosis or biomarker-based along the line of the A/T/N concept as A/T scheme (A, pathological Aβ42/40 CSF ratio; T, pathological CSF levels of total tau, indicative of neurodegeneration) (Jack et al., 2016). For parametric tests with adjustment for covariates (ANCOVA) and partial correlations, log-transformed values were used. The significance level was $\alpha = 0.05$. Principal component analysis was performed and visualized using BioVinci (BioTuring Inc.). Table 1 provides an overview of the numbers of subjects (N) per group within the different stratification schemes.

3 | RESULTS

3.1 | Relation to demographic & clinical features

An overview of demographic features and routine AD biomarker levels of the cohort and the patient groups by clinical diagnosis is provided in Table S1. Age, sex, and frequency of the *APOE* E4 genotype were unevenly distributed between the patient groups and therefore constituted relevant covariates for statistical analysis (Table S2). Ferritin and FABP-3 were elevated in MCI and AD against ND patients (Figure 1a,d,g, and statistical details in Table S3). Neurogranin was elevated in AD against ND, IPD, FTD, and ALS, and in MCI against FTD. All three markers were elevated in MCI with pathological amyloid ratio (MCI-A) against other MCI cases (MCI-O, Figure 1b,e,h). When adjusting for the potential covariates age, sex and *APOE* genotype, the elevation of ferritin in MCI-A and of FABP3 in MCI and AD were not robust against *APOE* genotype and age, respectively (indicated by red significance labels). All other tests based on clinical diagnosis were still significant after adjustment for covariates, of which age and sex were most influential (Table S3). Cognitive assessment data was available most of all for MCI and AD subjects in this cohort (Table S4). The candidate markers showed negative correlations to mini-mental state examination and free and cued selective reminding test (FCSRT) scores, representing worse cognitive outcome with higher levels of the candidate markers. However, most of these correlations did not pass adjustment for age as covariate of degeneration markers and cognitive performance, except for ferritin

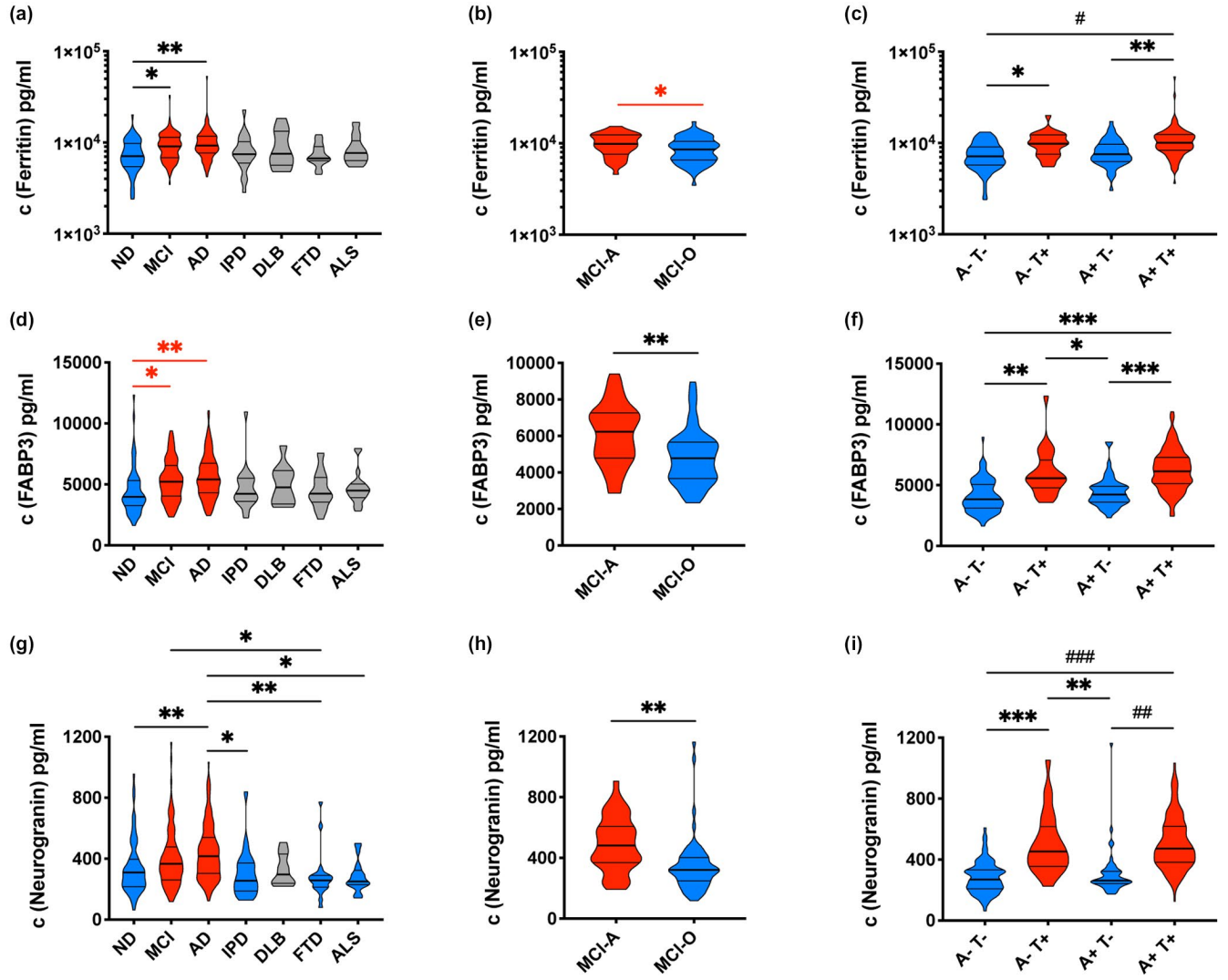

FIGURE 1 Group-wise comparison of ferritin, fatty acid binding protein 3 (FABP3), and neurogranin CSF levels. CSF concentrations of the three markers displayed as violin plots with median and interquartile range. Groups were based on stratification of either clinical diagnosis, amnestic (a) or other (o) mild cognitive impairment (MCI), or combined amyloid/tau biomarker positivity (A/T). Numbers of subjects (N) are provided in table 1 for each group or sub-group. (a–c) Ferritin, (d–f) FABP3, (g–i) neurogranin. Asterisks indicate level of significance of pairwise comparisons: $^{*}p < .05$, $^{**}p < .001$, $^{***}p < 1x10E^{-6}$, $^{\#}p < 1x10E^{-9}$, $^{\#\#}p < 1x10E^{-12}$, $^{\#\#\#}p < 1x10E^{-15}$. Red labels indicate comparisons not robust against covariates: (b) not robust against APOE genotype, (d) not robust against age. Groups colored red were elevated against at least one group in blue. Grey color indicates indifferent groups. Statistical details are provided in supplementary table 3. In line with previous findings, the three markers were elevated in MCI and Alzheimer´s disease compared to non-demented patients or patients with other neurodegenerative disorders, and also elevated in amnestic MCI. Yet, combined stratification by the A/T scheme indicates that these effects are driven by subjects with pathological tau levels independent of amyloid status

which retained a modest negative correlation to FCSRT score driven by the MCI cohort (Table S4).

3.2 | Influence of neurodegeneration markers

We next aimed to clarify whether changes in levels of ferritin, FABP3 or neurogranin in AD were driven by beta-amyloid accumulation or neurodegeneration (the latter assessed by CSF t-tau determination). Using a combined beta-amyloid/t-tau biomarker positivity scheme (A/T scheme), all three markers were elevated only in tau positive groups against tau negative groups, independent of the beta-amyloid status (Figure 1 C,F.I). This finding was robust against covariates for all markers (Table S3). Ferritin, FABP3, and neurogranin were also elevated within the tau positive subjects of other disorders (PD, DLB, FTD, and ALS), after exclusion of all AD, MCI, or ND cases (Figure 2). Application of the combined A/T scheme to the PD, DLB, FTD, and ALS groups was limited by sample size, however, there was still a trend for each of the three markers to be elevated within the tau positive subgroups of these non-AD spectrum disorders, again independent of the subjects' beta-amyloid status (Figure S1). Hence, all three candidate markers were considered as markers of neurodegeneration independent from beta-amyloid pathology, even if effects were more pronounced in AD compared to other NDs.

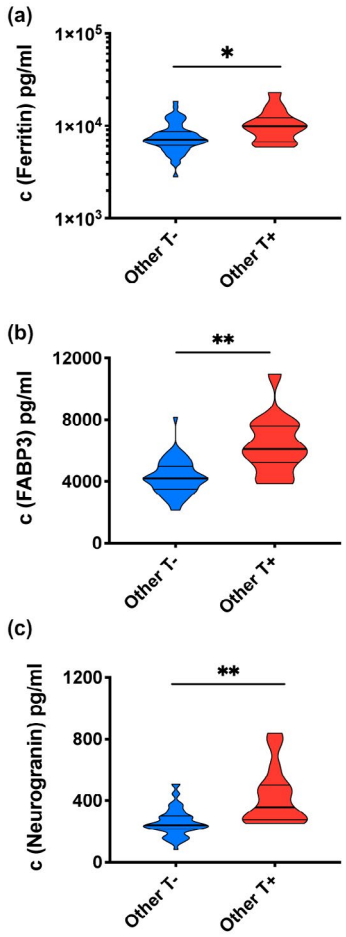

FIGURE 2 Other disorders display elevated levels in subjects with pathological tau biomarkers. Data of Parkinson´s disease (PD), DLB, ALS, and FTD patients was pooled and stratified by pathological (numbers of subjects $N = 15$) Vs. non-pathological ($N = 57$) t-tau levels. Violin plots with median and interquartile range, groups in red elevated against groups in blue. (a) ferritin, (b) fatty acid binding protein 3 (FABP3), (c) neurogranin. Asterisks indicate level of significance of pairwise comparisons: $^{*}p < .05$, $^{**}p < .001$. Despite the limited sample size, all three markers were elevated in those subjects which had pathological tau CSF levels independent of the diagnostic classification. Further stratification of these groups was limited by sample size, though trends (Figure S1) mirrored findings made for the whole cohort using the A/T scheme (Figure 1)

3.3 | Blood–brain barrier dysfunction does not relate to levels of the markers

As blood–brain barrier (BBB) dysfunction is prevalent in AD and other CNS NDs (Sweeney et al., 2018), we tested whether BBB dysfunction (measured by serum/CSF albumin ratio) had any influence on CSF levels of ferritin, FABP-3 or neurogranin. Data on BBB dysfunction was available for a fraction of all subjects (Table 1). Patients groups defined by diagnostic classification (ND, MCI, AD) or by A/T scheme (all samples including other disorders) were further subdivided into BBB dysfunction (positive) and BBB normal function (negative) sub-groups and statistically analyzed. BBB dysfunction did not influence results of any group-wise comparisons (Tables S5 and S6). Only for neurogranin in clinical stratification, there was a non-significant trend toward lower neurogranin levels in the ND and MCI groups for subjects positive for BBB dysfunction (Figure S2). Otherwise, there were no BBB dysfunction-dependent trends.

3.4 | Relation to inflammatory markers

To test the interrelations between ferritin, FABP3, neurogranin and markers of inflammation, we first calculated a PCA with the covariates age, sex and *APOE* status, AD biomarkers, inflammation markers, and the three candidate biomarkers for the ND, MCI, and AD patients (Figure 3, statistical details in Table S7, group-wise plots and 2-dimensional biplots in Figures S3 and S4). Separation of patients by clinical group (ND, MCI, AD) was significant for PC 1 to PC 5, whereas PC 6 and remaining PCs did not significantly differentiate between groups and accounted for less than 5% of variance in the dataset (supplementary table, Figure S3). The MCI group, including subjects at risk for AD and earliest in the time course of disease within this cohort, was separated from ND patients by PCs 1, 3, and 5. PC 3 was enriched for inflammatory markers, whereas in PC 5 classical AD hallmark markers or risk factors such as amyloid ratio, Age, or APOE status as well as further inflammatory markers were relevant. Ferritin, FABP3 and neurogranin were predominantly integrated into the 1st PC (PC 1), and to lesser extend into the other PCs. Despite the significant test results, there was still a clear overlap between patient groups in the scores of each PC. Visually, spread of patients was most pronounced along the axis of PC 1 (Figure 3, Figure S4). This PC furthermore included tau isoforms but also those inflammation markers previously described as highly related to CSF tau levels in this cohort [macrophage migration inhibitory factor, (MIF), soluble vascular endothelial growth factor receptor (sVEGF-R), sTREM2, soluble vascular cell adhesion molecule 1 (sVCAM-1), sICAM-1, and complement C1q) (Brosseron et al., 2018). To test the pairwise interactions of the three markers, we calculated a correlation matrix (Figure 4). Ferritin, FABP-3 and neurogranin were uniformly correlated with each other, tau isoforms, age, levels of Aβ40 - but not Aβ42 – and the same set of inflammatory proteins (Figure 4, all groups). These correlations were significant in subjects with or without pathological tau levels (Figure 4 Tau- and Tau+). Ferritin and FABP-3, but not neurogranin, showed furthermore weak correlations to Il-8, MCP-1, sIl-1RAcP, and C3aDesArg, but not VEGF, Il.6, SAA, or CRP. Neurogranin, but not ferritin or FABP-3, showed weak negative correlations to VEGF, Il-6, and CRP. Most of these weaker correlations became insignificant if the cohort was stratified by pathological/nonpathological t-tau levels. Overall, there was no obvious pattern of strong correlations that would appear only in subjects with or without neurodegeneration.

The bivariate matrix did not provide information on the actual trajectory (steepness and intercept) of the correlations. We therefore tested whether the trajectory of the correlations between ferritin, FABP-3 or neurogranin on the one hand and the five most

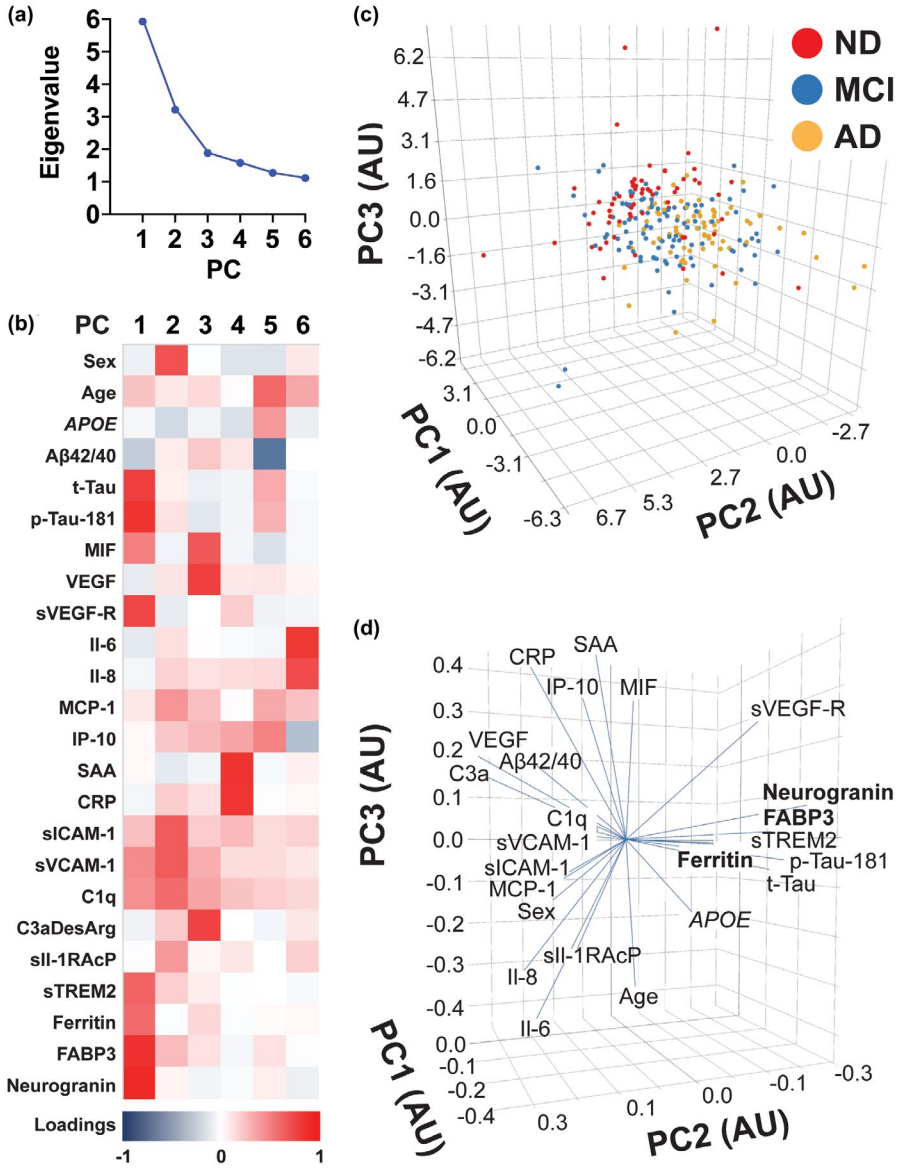

FIGURE 3 Principal component analysis neurodegeneration and inflammation markers. PCA was computed using levels of ferritin, fatty acid binding protein 3 (FABP3), and neurogranin together with amyloid and tau markers, covariates (age, sex, *APOE* E4 positivity) and inflammation biomarker data for all ND, mild cognitive impairment (MCI), and Alzheimer´s disease (AD) subjects (number of subjects $N = 283$). In total, 24 variables were included in the analysis. (a) Eigenvalues > 1 were reached by the first 6 principal components (PCs), indicative of PCs that represent more variance than a single variable. (b) Composition loadings of the PCs displayed as heat map. Within the 1st PC, tau isoforms and the three candidate markers were most influential, as well as those inflammation markers previously described to be strongest related to tau levels [e.g. macrophage migration inhibitory factor (MIF), sVEGF-R, sTREM2]. Ferritin, neurogranin, and FABP3 were barely relevant in other PCs. (c) Three-dimensional display of ND/MCI/AD-patient data within the first three PCs. Patients groups were separated strongest by the 1st PC. (d) Vector graphic of single variables within the first three PCs. Vectors of the three candidate markers are highlighted bold. Vectors are in line with tau isoforms, but also sTREM2

significantly correlated inflammation markers (MIF, sVEGF-R, sVCAM-1, C1q, and sTREM2) on the other hand differed between individuals with normal or pathological tau levels (Table S8 and Figure S5). This was not the case for most correlations of FABP-3 and neurogranin. FABP-3 had only one differing correlation to sVCAM-1 that was on slightly elevated track in non-pathological individuals (Figure 5 H). For neurogranin, only the correlation to sVEGF-R differed between individuals with or without neurodegeneration and was on slightly steeper track in non-pathological individuals (Figure 5i). In contrast to FABP-3 and neurogranin, ferritin showed differences in the correlations to all relevant inflammation markers: Relations to sVEGF-R, sVCAM-1, C1q, and sTREM2 were steeper in non-pathological than in pathological individuals (Figure 5c–f). For MIF, the correlations to both ferritin and neurogranin were on elevated track in individuals with pathological tau levels (Figure 5 G and J).

Ferritin, neurogranin, and FABP-3 as were furthermore correlated with age (Figure 4), and so where the correlated inflammatory markers (Brosseron et al., 2018). We therefore tested whether the interactions between inflammation and neurodegeneration markers were robust against age as covariate (Table S9). In brief, this was the case for all correlations between the three candidate markers and inflammation markers, though the strength of the correlations was reduced. Ferritin correlations were least robust against age, neurogranin most robust. Of the inflammation markers as correlates, sVEGF-R was most robust against age, with less reduction in strength as compared. to other inflammatory proteins.

Vice versa, correlations between age and inflammatory proteins can be on altered trajectory in individuals with neurodegeneration measured by t-tau levels (Brosseron et al., 2019). As ferritin, FABP3 and neurogranin likewise constitute neurodegeneration markers, we tested whether such inflam-aging correlations could be differentiated by these three markers in similar manner as by pathological t-tau levels. For this purpose, we first determined cut-off values for pathological (high) levels of the three markers based on discrimination of both AD Vs. ND subjects as

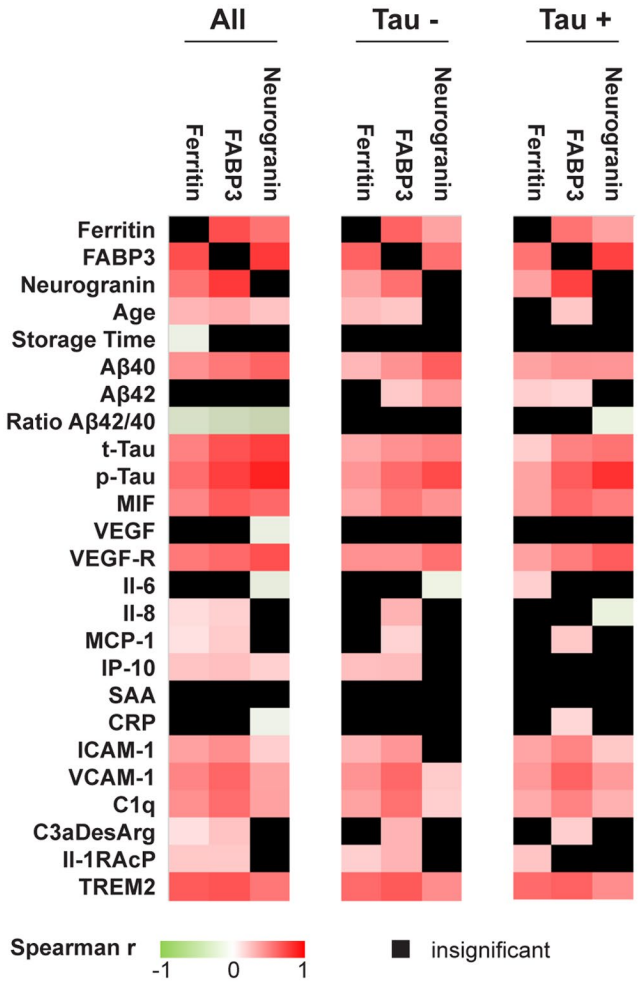

FIGURE 4 Correlation matrices for the three candidate markers between subjects with or without neurodegeneration. Spearman r values displayed as heat map for correlations between ferritin, fatty acid binding protein 3 (FABP3), neurogranin and standard Alzheimer´s disease (AD) biomarkers, inflammation markers, and covariates. Overall ND, mild cognitive impairment, and AD (number of subjects $N = 283$), the strongest correlates were tau isoforms, macrophage migration inhibitory factor (MIF), sVEGF-R, and sTREM2. Strength of correlations to neurodegeneration markers increased from ferritin over FABP3 to neurogranin. For inflammation markers, relations where more differentiated, as ferritin or FABP3 showed stronger relations to some markers [e.g. soluble vascular cell adhesion molecule 1, C1q, or sTREM2] then neurogranin. Overall, there was strong parallelism in correlations of the three markers: Frequently, these were either all related to other markers or all not. When the cohort was split into tau positive ($N = 150$)/ negative subjects ($N = 133$), there was no striking change in strength of these correlations. The three markers correlated modestly to age (see supplementary table 2). Only for ferritin there was a weak negative correlation to storage time of samples at −80°C, which was only significant in the large dataset of all cohort samples

well as pathological versus non-pathological t-tau or p-tau-181 levels (Table S10). The discriminatory power for the three markers in these models with equally weighted sensitivity/specificity was 62.5%–66.0% (ferritin), 65.0%–78.0% (FABP3), and 65%–84.8% (neurogranin). Discriminatory power was always lowest for discrimination of AD versus ND cases, and higher for discrimination based on t-tau and p-Tau-181. As cut-off values, 8,500 pg/ml (ferritin), 5,000 pg/ml (FABP3), and 350 pg/ml (neurogranin) were used (Table S10). On this basis, we then tested whether inflam-aging correlations of MIF, sVEGF-R, sVCAM-1, C1q, or sTREM2 differed between individuals with elevated levels of ferritin, FABP3, or neurogranin (Table S11). There were only two significant differences when dichotomizing samples by pathological ferritin levels (Figure 5a,b): sVCAM-1 was only correlated with age in subjects with low ferritin levels. For higher ferritin levels, the correlation was on different intercept but without significant steepness. C1q was correlated to age in all subjects, but with higher trajectory in those with pathological ferritin levels.

Next, we tested if the three candidate markers were related to inflammation markers independent of neurodegeneration by adjusting against t-tau or p-tau-181 (Table S12). Most correlations between the candidate markers and inflammation markers remained significant after adjustment but clearly lost in strength. Vice versa, when adjusting the correlations between ferritin, FABP3, or neurogranin with t-tau or p-tau-181, respectively, against the levels of inflammation markers, the correlations between the candidate markers and tau isoforms remained highly significant with limited loss in strength (Table S13).

Finally, we compared the steepness of the correlations between tau and the 3 markers as well as inflammation markers with p-tau-181 as reference (Figure 5k). Of the three candidate markers, neurogranin showed the steepest correlation to tau. FABP3, ferritin and the inflammation markers all showed a less steep increase against t-tau, and all in similar effect size.

4 | DISCUSSION

4.1 | Ferritin, FABP-3, and neurogranin as neurodegeneration markers

Within the spectrum of NDs, the three proteins investigated in this study have been previously reported primarily as markers of AD. In the cohort tested in this study, we also observed elevation of the three markers in AD and MCI, in particular in beta-amyloid positive MCI cases (Figure 1). At first glance, this is in line with previous findings for the three markers and apparently specific for the AD disorder spectrum (; Chiasserini et al., 2017; Diouf et al., 2019; Guo et al., 2013; Kester et al., 2015; Kvartsberg, et al., 2015; Lista et al., 2017; Mattsson et al., 2016; Olsson et al., 2013, 2016; Pereira et al., 2017; Portelius et al., 2015; Sanfilippo et al., 2016; Steinacker et al., 2004; Sun et al., 2016; Tarawneh et al., 2016; Thorsell et al., 2010; Wang & Alzheimer's Disease Neuroimaging Initiative, 2019; Wellington et al., 2016, 2018). The candidate markers were also negatively correlated with mini mental state examination and FCSRT scores, albeit this was not robust against age as covariate in most cases (Table S4).

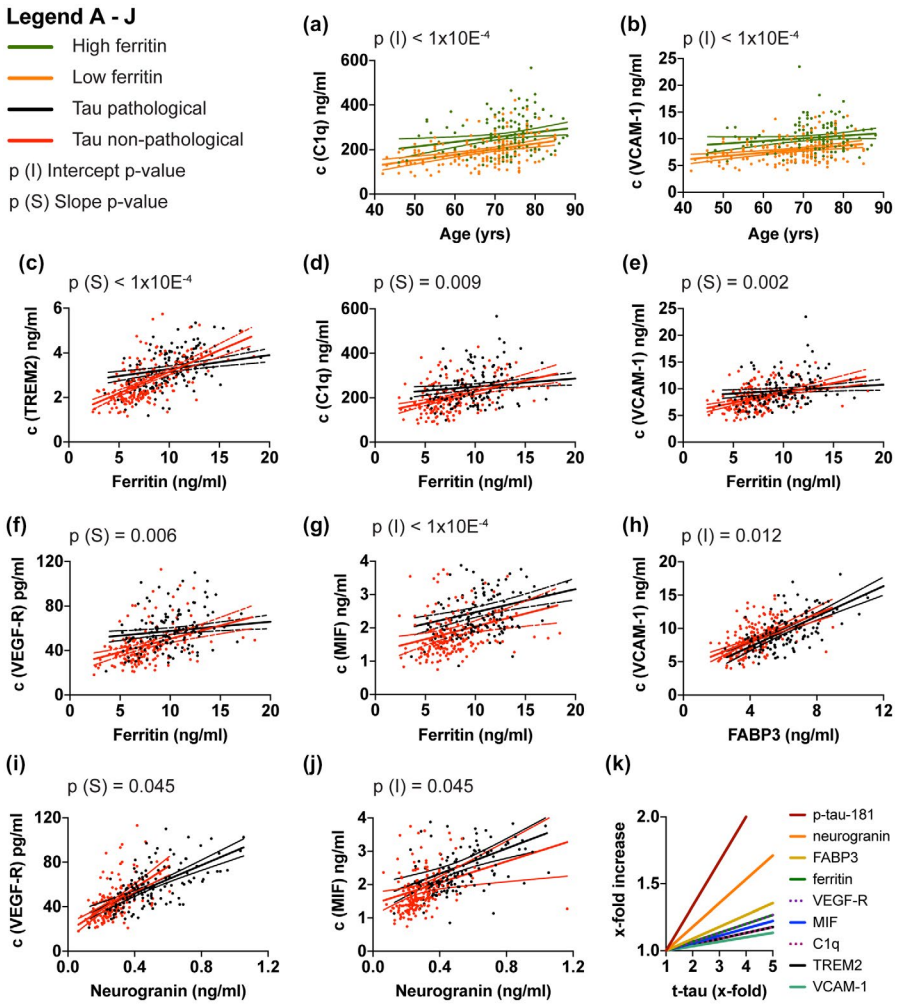

FIGURE 5 Interrelations between age, neurodegeneration, and inflammation markers. Display of significant findings from test on relations to inflame-aging and between neurodegeneration and inflammation. Correlations with different slope (S) or different intercept (I) are displayed in color-code with p-values and 95% confidence intervals. (a, b) Elevated levels of ferritin, fatty acid binding protein 3 (FABP3) or neurogranin did not dichotomized the correlations between inflammation markers and age, with two exceptions: correlations between age and either C1q or soluble vascular cell adhesion molecule 1 (sVCAM-1) were on elevated track in subjects with higher ferritin levels (number of subjects $N = 151$) against subjects with lower ferritin levels ($N = 132$). Ferritin showed also more differences in correlations between subjects with or without neurodegeneration (dichotomized by t-tau levels, $N = 150$ tau positive, $N = 133$ tau negative): Correlations between ferritin and sTREM2 (c), C1q (d), sVCAM-1 (e), and sVEGF-R (F) differed in slope, with steeper correlations in non-pathological subjects. In pathological subjects, these were still correlated, but with a rather flat trajectory. Only a correlation between ferritin and macrophage migration inhibitory factor (MIF) (g) did not change steepness in pathological subjects, but instead was on elevated trajectory. There was only one significant finding for the correlation between sVCAM-1 and FABP3 that was on slightly elevated track in pathological subjects (h). For neurogranin, correlations to sVEGF-R (i) and MIF (j) differed between pathological/non-pathological subjects in similar manner as for ferritin, but less pronounced. In relation to t-tau, neurogranin showed the steepest correlation, whereas FABP3, ferritin, and inflammation markers showed more modest correlations (k)

Yet, when applying the A/T scheme for stratification, it was evident that the effects observed for MCI subtypes or AD were actually driven by individuals with pathological levels of CSF total tau, representative for neurodegeneration, and independent of beta-amyloid status (Figure 1). Tau-based stratification resulted in more significant differences compared to clinical classification. Elevation in tau positive subjects was furthermore much more robust against covariates such as age, sex, or *APOE* status, and there was also no indication of potential influence of BBB dysfunction even if just by trend. This strong relation to tau was also observed in the correlation matrices and PCA analyses (Figures 3 and 4) and has been described by others before, in particular for neurogranin and FABP-3 (Bjerke et al., 2016, 2011; Kester et al., 2015; Kvartsberg, et al., 2015; Lista et al., 2017; Mattsson et al., 2016; Olsson et al., 2013; Portelius et al., 2015; Sanfilippo et al., 2016; Sun et al., 2016; Tarawneh et al., 2016; Thorsell et al., 2010; Wang & Alzheimer's Disease Neuroimaging Initiative, 2019; Wellington et al., 2016, 2018). Previously reported relations of the three candidate proteins to amyloid are likely to be a by-product of this relation to tau. Use of other stratification approaches that do not include tau status are therefore misleading.

Interpretation of the candidate markers as neurodegeneration markers is in line with the localization of neurogranin and FABP-3, which are intracellular proteins and likely to be released as consequence of synaptic or neuronal damage and degeneration. Interpretation of ferritin is more challenging. Ferritin as an iron binding protein is ubiquitously expressed; both mechanistically and as a CSF marker ferritin has been related to various CNS disorders and conditions, including traumatic brain injury, and might in the end constitute a sensitive, but not very specific marker of a range of CNS pathologies (Finazzi & Arosio, 2014; Kolodziej et al., 2014; Ondruschka et al., 2018; Russell et al., 2019). Under pathological conditions, extracellular ferritin could be derived from different cellular sources. In example, ferritin can be released by dying neurons, or by microglia that react to increased and potentially cytotoxic levels of extracellular iron (Thomsen et al., 2015). Brain iron load correlates with tau-PET uptake as well as cognitive decline, in particular in subjects that already present with AD pathological hallmarks plus onset of symptoms (Ayton et al., 2019; Spotorno et al., 2020). These findings were interpreted to be in line with increases in in iron during the neurodegeneration and, in consequence cognitive symptomatic phase of the disease, after accumulation of the pathological hallmarks. In contrast, subjects with AD pathology but without cognitive symptoms did not show such correlations to brain iron load. The exact mechanism behind this co-occurrence of neurodegeneration and iron accumulation still remains to be fully elucidated (Masaldan et al., 2019). Intracellular iron accumulation is part of the phenotype of cellular senescence (Masaldan et al., 2018). Accumulation of iron within the brain might be a consequence of neuronal degeneration and the subsequent iron release from the dying neurons, but also foster this process, for example, by induction of oxidative stress or ferroptosis. Vice versa, toxic iron accumulation might be related to other pathological mechanisms like vascular dysfunction or neuroinflammation and constitute a turning point that induces neurodegeneration in tissue with already prevalent AD pathology. Findings for ferritin CSF levels are in line with these observations on brain iron accumulation and are likely to be influenced by multiple iron-related mechanisms that accumulate at the stage of onset of neurodegeneration and cognitive symptoms. Hence, ferritin could represent both a direct as well as a responsive marker of neuronal degeneration within the spectrum of NDs.

Some previous studies have also reported elevated levels of the three markers in non-AD spectrum NDs, too (Bereczki et al., 2017; Bjerke et al., 2011; Chiasserini et al., 2017; Matsui et al., 2010; Zheng et al., 2017). In example, neurogranin is negatively correlated with cognitive function and predictive of cognitive decline. In other NDs such as frontotemporal dementia (FTD) or Parkinson´s disease (PD), neurogranin is not reported to be elevated, though sub-groups of PD patients might show differences (Bereczki et al., 2017). Shioda et al. found FABP-3 to be involved in dopaminergic neuron degeneration and α-synuclein oligomerization in Parkinson´s disease animal and cell culture models (Shioda et al., 2014).

In our cohort, we did not observe elevation of one of the markers in other disorders then AD. This difference might be because of the limited sample size of non-AD spectrum disorders within our dataset, or specific subtypes of other disorders not represented in our cohort. However, if subjects with other NDs had pathological tau levels, these had likewise higher levels of ferritin, FABP-3 or neurogranin (Figure 2). By trend, this was also observed when applying the A/T scheme to the other disorders despite limitations in sample size, in line with independence of the amyloid status (Figure S1).

In conclusion, although elevation of the three markers is most pronounced in subjects in the AD spectrum, ferritin, FABP-3 and neurogranin all represent markers of neurodegeneration. Among the three, neurogranin was the strongest marker, followed by FABP-3, and ferritin was least strong in all types of correlation analysis, group wise comparisons or discriminative power analysis. This is relevant for applicability of the three proteins as biomarkers in studies, interpretation of data, and might also reflect differences in their biology in particular for ferritin (see below).

4.2 | Relation of the three candidate markers to inflammation

It was expected that if ferritin, FABP-3 and neurogranin constitute biomarkers of neurodegeneration, inflammatory markers previously described as related to t-tau as standard marker of neurodegeneration should be likewise related to the three candidate markers. This was indeed the case for MIF, sVEGF-R, sTREM2, sVCAM-1, sICAM-1, and complement C1q, all of which were related to the candidate markers in PCA as well as bivariate correlation analysis (Figures 3 and 4). All of these inflammation markers were positively correlated with ferritin, FABP-3, and neurogranin, and this was significant in subjects with or without pathological levels of total tau (Figure 4). For FABP-3 and neurogranin, most correlations to inflammation markers did not differ in intercept or steepness between subjects with pathological or nonpathological tau levels, though lost significantly in strength if statistically adjusted for t-tau. This may suggest that the relation of any inflammatory damage response to neurogranin and FABP-3 is independent of the absolute extend of damage.

In contrast, for ferritin these interactions were more complex: When dichotomizing inflammation-to-aging correlations using the cut-offs for pathological levels of the three markers, only ferritin had a noteworthy impact (Figure 5): In subjects with higher ferritin levels, age correlations of C1q and sVCAM-1 were on elevated trajectory. This finding is similar to previous results with t-tau as dichotomizing factor for correlations of inflam-aging (Brosseron et al., 2019). However, there was no such effect for any other age-correlated inflammation marker, such as sTREM2 or MIF, and ferritin was a weak marker of neurodegeneration when compared to tau, neurogranin or FABP3. To our knowledge, there is no direct mechanistic interaction described between ferritin and either C1q or sVCAM-1, and it is possible that the levels of these proteins are in the end independently affected by other factors than the response to neuronal damage, such as age-dependent physiological changes that affect iron level regulation or inflammation, respectively.

Furthermore, the direct bivariate correlations between ferritin and the inflammation markers differed between individuals with or without pathological t-tau levels: Although these relations were of similar strength and significance in both types of individuals, they differed either in steepness or intercept (Figure 5). In individuals with neurodegeneration, the correlations between ferritin and sTREM2, C1q, sVCAM-1 and sVEGF-R lost in steepness. This indicates that levels of ferritin and the respective inflammation factors are co-regulated under physiological conditions, but not anymore or to significantly lesser extend in individuals that experience neurodegeneration. In contrast to FABP-3 and neurogranin, ferritin is secreted under non-pathological conditions and expressed by various cell types in the CNS. Ferritin in the periphery can be elevated during acute phase response or vascular disorders, which could explain the co-regulation of ferritin with C1q, sVEGF-R or other inflammatory proteins under non-pathological conditions. At the same time, ferritin levels rise in response to neuronal damage, including release from dying neurons or microglia. In this study, the increase in CSF levels of ferritin was weaker than that of FABP-3 or neurogranin, but still stronger than that of most inflammatory markers (Figure 5k). The different mechanisms of ferritin release might be the reason behind the changes in ferritin correlations between individuals with or without neurodegeneration. In contrast, neurogranin and FABP-3 are not secreted under physiological conditions and should be considered as more specific markers of synaptic or neuronal degeneration. As such, they relate to inflammation markers in similar manner as tau isoforms or Nf-L.

4.3 | Differences between inflammation markers in relation to ferritin, FABP3 or neurogranin

The interrelation analysis revealed not only differences between the three markers toward inflammatory proteins, but also between the inflammatory proteins in relation to the candidate markers. Some inflammatory factors, like Il-8, IP-10 or MCP-1, were only very modestly related to any of the three neurodegeneration markers. Several others (e.g. VEGF, Il-6, SAA, or CRP) were barely related to ferritin, FABP3, or neurogranin at all. Correlations between MIF and neurogranin and—more significantly—ferritin did not differ in steepness between individuals with or without neurodegeneration, in contrast to the other correlated inflammation markers. Instead, MIF levels were in general higher in individuals with neurodegeneration, but still correlated with ferritin in the same way as in non-pathological sindividuals. This could indicate that there is co-regulation between MIF and ferritin under both physiological conditions as well as—in increased manner for both—during neurodegeneration. Such co-regulation between ferritin and MIF levels has been observed before in serum of subjects with adult-onset Still's disease (Becker et al., 2009) and might be caused by underlying pathological conditions. There is little mechanistic data regarding an interaction between MIF and ferritin, but murine macrophages up-regulate both proteins (among others) in response to extracellular iron (Polati et al., 2012). Potentially, this is of relevance for microglial iron control, too, though to our knowledge there is currently no information on any specific functions of MIF in iron regulation.

4.4 | Limitations

There are some limitations to this study that are of relevance for interpretation. First, the cohort analyzed here is derived from a neurological outpatient clinic. This has the advantage of being very close to daily practice of clinicians. However, this cohort cannot provide entirely healthy controls, as the non-demented comparator patients in this cohort are not without disorders such as peripheral neuropathies, subjective cognitive decline or normal pressure hydrocephalus without cognitive impairment (Brosseron et al., 2018). Furthermore, earliest stages of dementia or AD, such as subjective cognitive decline, are not represented here. The limited discriminative power of the investigated markers, compared to established neurodegeneration markers like tau, might be stage-dependent, and the markers might have more potential at earlier stages. Second, this cohort does not include longitudinal assessments, though some studies have described predictive potential of the three markers investigated here. Further studies are hence required to compare the data presented here to earlier stages of disease development in which the markers might show higher potential, and in which the interrelations to inflammatory markers might be different, too.

4.5 | Outlook

This study provides further evidence that a specific set of proteins is highly correlated with various markers of neurodegeneration. Of the three candidate markers, FABP-3 and neurogranin clearly represent such degeneration markers as they are elevated independent of amyloid status, and categorization as neurodegeneration marker is in line with their cellular localization. The third candidate marker, ferritin, follows a similar pattern of changes in CSF levels, but could be released by different cell types, including – but not limited to – neurons. The inflammatory markers highly related to these and other markers of neurodegeneration probably represent a readout of the inflammatory response to the neuronal death in AD and other degenerative disorders of the CNS. Within this group of markers, sTREM2, MIF, sVEGF-R, sVCAM-1, and C1q represent the most significant and promising candidates for biomarker panels to monitor this response. Such a panel might not trace the very earliest inflammatory responses that accompany accumulation of pathological protein aggregates, but could nonetheless serve as readout for those immune mechanisms that respond to neuronal death. As the relations described in this work where largely independent of the extend of this damage, it is likely that this type of inflammatory response still occurs relatively early throughout the course of disease, for example, in subjects that are asymptomatic or only subjectively symptomatic but already present with a pathological neurodegenerative

biomarker profile. If these individuals can be identified, treatments targeting the inflammatory response may be tested still before onset of major symptoms of dementia. Blood-based tests for neurodegeneration markers like Nf-L or tau isoforms are on the rise and future study designs will include such measures to screen for individuals with early CNS pathology. This group of individuals then is likely to be relevant for immunological therapeutic intervention at this stage of disease. Further characterization of the inflammatory biomarkers most promising for monitoring, the exact mechanisms behind their regulation, their trajectory throughout disease and interactions with other pathological features constitute the next steps toward application in clinical trials and studies.

4.5.1 | Author´s contributions

FB contributed to conception of the study, design of the work, acquisition, analysis and interpretation of data, and drafted the work. KK contributed to analysis and interpretation of data and drafted the work. FS, CCK, and EL contributed to design of the work and acquisition of data. SCG and PT contributed to provision of human biomaterial and subject's clinical data. MTH contributed to conception of the study, interpretation of data, and drafted the work. All authors read, revised, and approved the manuscript before submission.

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CONFLICT OF INTEREST

Michael T. Heneka holds editorship at the Journal of Neurochemistry. The authors declare no further competing interests.

DATA AVAILABILITY STATEMENT

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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