frontiers | Frontiers in Lupus

TYPE Original Research
PUBLISHED 14 May 2024
DOI 10.3389/flupu.2024.1386256

OPEN ACCESS

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RECEIVED 14 February 2024
ACCEPTED 29 April 2024
PUBLISHED 14 May 2024

CITATION
Wegener MAB, Möller S, Olesen MN, Madsen JS, Sorensen GL, Voss A and Asgari N (2024) Microfibrillar-associated protein 4 interaction with inflammation and clinical characteristics in neuropsychiatric systemic lupus erythematosus.
Front. Lupus 2:1386256.
doi: 10.3389/flupu.2024.1386256

Microfibrillar-associated protein 4 interaction with inflammation and clinical characteristics in neuropsychiatric systemic lupus erythematosus

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Objectives: Central nervous system (CNS) proteins such as neurofilament light chain (NfL) and glial fibrillary acidic protein (GFAP) are released into the body fluids following CNS injury. Microfibrillar-associated protein 4 (MFAP4) is an extracellular matrix protein. Recently we reported expression of MFAP4 in CNS and alteration of levels in patients with acute neuroinflammation. We aimed to determine the levels of MFAP4 in a predominantly population-based cohort of systemic lupus erythematosus (SLE) patients, including neuropsychiatric SLE (NPSLE), and to evaluate MFAP4 as a marker of inflammation.

Methods: In total 208 SLE patients, 44 of those with NPSLE, and 50 age- and sex-matched healthy controls (HC) were recruited. MFAP4 was measured using AlphaLISA immunoassay. NfL, GFAP and a panel of inflammatory mediators were measured using Simoa HD-1 digital ELISA or a Luminex 200 instrument.

Results: MFAP4 levels were elevated in patients with NPSLE compared to patients with non-NPSLE ($p = 0.031$), more prominent in NPSLE patients with CNS involvement ($p = 0.017$). NfL and GFAP were higher in the total SLE cohort ($p < 0.001$, $p < 0.001$, respectively) as well as NPSLE subgroup ($p < 0.001$, $p < 0.001$, respectively), and in the subgroup of NPSLE patients with CNS involvement ($p < 0.001$, $p < 0.001$), compared to HC. NfL and GFAP levels correlated positively with MFAP4 in the NPSLE as well as the non-NPSLE subgroup ($\rho = 0.44$, $p = 0.003$, $\rho = 0.25$, $p = 0.004$). VEGF was reduced in NPSLE patients compared to HC ($p = 0.015$). MMP-9 was elevated in NPSLE compared to non-NPSLE ($p = 0.048$). Inflammatory markers including IFN-α, IL-6, IL-10 and TNF-α, were elevated in the NPSLE group compared to HC ($p < 0.001$, $p = 0.0026$, $p = 0.042$, $p = 0.007$, respectively). In NPSLE patients the levels of MFAP4 correlated with TNF-α ($p = 0.016$) and IL-17 ($p = 0.0044$) and with markers of blood brain barrier (BBB) disruption MMP-7 ($p = 0.005$) and VEGF ($p < 0.001$). In NPSLE patients with CNS manifestations MMP-3 and VEGF correlated with MFAP4 ($p = 0.011$, $p = 0.0004$, respectively).

Conclusion: Levels of MFAP4 correlated with NfL, GFAP and proinflammatory cytokines and in NPSLE additionally with markers of BBB disruption, suggesting that MFAP4 is a marker of inflammation and vascular re-organization. Correlation of NfL and GFAP with MFAP4 may reflect CNS tissue damage.

KEYWORDS

systemic lupus erythematosus, neuropsychiatric SLE, inflammation, microfibrillar-associated protein 4, accumulated disease damage

1 Introduction

Systemic lupus erythematosus (SLE) is an autoimmune, systemic inflammatory disorder with a heterogeneous clinical presentation (1, 2). It affects multiple tissue and organ systems such as skin, joints, serosa, blood vessels, kidneys and nervous system (1–3). In Denmark the estimated prevalence of SLE is 45.2 per 100.000 but globally up to 517.5 cases per 100.000 have been reported (4, 5). Certain genetic polymorphisms in combination with environmental exposures are believed to induce and maintain the disease (1, 3, 6). This interplay between genetic factors and environment leads to activation of both innate and adaptive immune system components (1), resulting in production of proinflammatory cytokines e.g., type 1 interferons (IFN), synthesis of autoantibodies that recognize the patients' own DNA or RNA, immune complex formation, and amplification of autoreactive lymphocytes (1, 3, 7). These mechanisms lead to a loss of tolerance to self-antigens and eventually lead to irreversible tissue and organ damage (1, 3).

Neuropsychiatric systemic lupus erythematosus (NPSLE) contributes considerably to morbidity and mortality in patients with SLE (8). NPSLE encompasses manifestations from both the central nervous system (CNS) and the peripheral nervous system (PNS) and may give rise to both neurological and psychiatric symptoms (8, 9). In 1999, the American College of Rheumatology (ACR) summarized these NPSLE manifestations and outlined 19 neuropsychiatric syndromes, 12 connected to the CNS and 7 to the PNS, that can appear in patients with SLE (8, 9). These manifestations were further subdivided into focal NPSLE such as stroke and non-focal/diffuse NPSLE such as psychosis, cognitive dysfunction and affective disorders (8, 9).

The exact prevalence of NPSLE is unknown but data indicates that more than 50% of SLE patients have CNS involvement with the most frequent manifestations being headache, mood disorders and cognitive dysfunction (8, 10, 11). Several studies have proposed different potential mechanisms to explain the pathogenesis of NPSLE involving proinflammatory cells, autoantibodies, cytokines, chemokines, complement and other molecules (8, 12, 13). Notably, type 1 IFNs have been found to be elevated in both serum and the hippocampus of SLE patients (8, 14). One specific effect of IFN-α is the production of several cytokines, including the proinflammatory interleukin (IL)-6 (13). IL-6 is found to be elevated in the serum and the CSF of NPSLE patients (13–15) and has been suggested to play a role in the breakdown of the blood-brain-barrier (BBB) in NPSLE (15). Disruption of the BBB is proposed as the common foundation for development of NPSLE as it may lead to entry of proinflammatory cells, cytokines and autoantibodies into the CNS causing neuroinflammation (8, 13, 16, 17). Thus, markers that mirror BBB disruption may be indicators of NPSLE (9) and also elucidate disease pathogenesis. In addition, neuronal and astrocytic damage as reflected in high intrathecal levels of neurofilament light chains (NfL) and glial fibrillary acidic protein (GFAP) have been reported in NPSLE with CNS involvement (18, 19).

Microfibrillar-associated protein 4 (MFAP4) is an extracellular matrix (ECM) protein belonging to the fibrinogen-related domain (FReD) family that includes different proteins engaged in tissue homeostasis and innate immunity (20–22). MFAP4 has binding affinity for the ECM components elastin and collagen that forms elastic and collagen fibers, and MFAP4 is found to play an active role in elastic fiber formation (20, 21, 23). Elastic fibers are ECM macromolecules that have the biomechanical properties of elasticity and resilience (20, 24). These properties are crucial for the structural integrity of the ECM and hence for the function of connective tissue e.g., in arteries, lungs, and skin (20, 24, 25). MFAP4 is measurable in serum, serum levels increase in specific conditions characterized by aberrant tissue remodeling (26) while ECM-bound MFAP4 is predominantly located in arteries and arterioles in most tissues (17). Very recently our group reported data on expression of MFAP4 in the CNS and alteration of the levels of soluble MFAP4 in cerebrospinal fluid (CSF) in patients with acute neuroinflammation and relapse (27). These data suggest that MFAP4 may serve as a potential biomarker of disease activity. Nonetheless, the potential role of MFAP4 in BBB integrity in patients with NPSLE is so far unexplored.

We propose that levels of MFAP4 in serum (a) may differ between SLE patients with and without NPSLE as well as healthy controls (HCs) and (b) may act as a biomarker for inflammation. Hence, this study aimed at measuring serum MFAP4 in a predominantly population based SLE cohort as well as healthy controls (HC), and correlate this to clinical characteristics, cytokines, and markers of BBB disruption.

2 Materials and methods

2.1 Study population

Patients: A clinical database with associated biobank was established for SLE patients diagnosed in the period 1995–2016

in the Region of Southern Denmark (28–30). A total of 208 SLE patients were included, with 124 cases originating from a population-based cohort from Funen and 84 cases from a clinic-based cohort from the rest of the Region of Southern Denmark as described previously (28, 30). All 208 patients, primarily white Europeans (98%) aged 18–70 years at present and with a female to male ratio of 6.4:1, were included in this cross-sectional study (28, 30). As described previously patients with NPSLE were classified in this SLE cohort (30) in accordance with the ACR nomenclature and case definitions for neuropsychiatric SLE syndromes (31–34, 35), and 44 NPSLE patients were included in the present study, 37 of 44 (84.1%) with CNS involvement (30). Patients not classified with NPSLE are denoted as non-NPSLE in this study. Clinical characteristics and treatment status reported was collected between 2010 and 2012 except patients' NPSLE status which was classified in 2017. Each patient was diagnosed with SLE by the occurrence of multisystem disease, autoantibodies, and exclusion of other diseases according to Fries & Holman (28, 31) and was classified according to the 1997 revised ACR criteria (28, 32, 33). At the time of inclusion patients had to be 18 years or older, and patients with primary antiphospholipid syndrome and drug induced SLE were excluded from the study (28, 29). Disease activity was monitored using the SLE Disease Activity Index 2000 (SLEDAI-2K) (36). Cumulative disease damage was calculated using the Systemic Lupus International Collaborating Clinics/American College of Rheumatology Damage Index for SLE, SLICC (28, 33, 37, 38) reflecting both damage due to disease and treatment of SLE (33, 37, 38). The clinical profile of the SLE cohort is comparable to other Western European study populations and has previously been described (28–30).

Controls: Serum from fifty self-reported healthy anonymized blood donors aged 18–65 years was obtained from the blood bank at the Department of Clinical Immunology, Odense University Hospital, Odense, Denmark, in January 2021. Healthy controls (HCs) were from the same geographical background population as the SLE cohort and were matched with regard to age and sex.

2.2 Ethics

The study complies with the Declaration of Helsinki. The clinical data material and blood samples were collected in a study approved by The Regional Committee on Biomedical Research Ethics (Ref. no. S-20100015). Before donation each blood donor approved the use of their blood for research purposes in line with relevant national guidelines (*Standards in Transfusion Medicine*).

2.3 Laboratory analyses

Patient serum was obtained from blood samples taken from 2010 to 2012. Blood samples were collected using standard venipuncture and allowed to clot at room temperature for 30 min before centrifuged for 10 min, serum was transferred into cryo-tubes and stored at −80°C until laboratory analysis. Blood samples from HC were taken in 2021 by venipuncture and stored overnight for coagulation at 4°C before being centrifuged, aliquoted and stored at −80°C. This cold overnight storage has been shown not to affect levels of various cytokines (39).

Serum MFAP4 was measured using AlphaLISA immunoassay (Perkin Elmer, MA, USA) as formerly described (20). Samples were run in duplicates, and occasional samples with coefficient of variation (CV) > 10% (2/208 samples 0.96%) were re-analyzed to obtain a valid measurement for every sample.

Inflammatory mediators in serum were measured on a Luminex 200 instrument (Luminex Corporation, TX, USA, custom designed panel) using a 7-plex kit for interleukin (IL)-6, -10, 17, matrix metalloproteinase (MMP)-3, -7, vascular endothelial growth factor (VEGF) and tumor necrosis factor (TNF)-α and 2-plex for MMP-2 and −9 according to manufacturer's instructions (premixed Human Magnetic Luminex Assay, R&D Systems, MN, USA) (40). Due to limited materiale available MMP-2 and -9 were analysed in 176 out of the 208 SLE patients. All Luminex analyses were done at the Department of Clinical Immunology, Odense University Hospital, Denmark, in February and March 2021.

Serum concentrations of IFN-α, NfL and GFAP were analyzed at the Department of Biochemistry and Immunology, Lillebaelt Hospital, Vejle using the commercial available 2-plex assay for the Single molecule array (Simoa) HD-X Analyzer (Quanterix, Billerica, MA, USA, NfL and GFAP no. 1035209, IFN-α, no.100860), according to manufacturer's instructions (41, 42). The total analytical variation for the included controls were 10%–16% for NfL and 8%–14% for GFAP.

In-house quality controls consisting of pooled anonymous sera were used as inter-run quality control in Luminex (CV: 32% for IL-6, 15% for IL-17, 31% for TNF-α, 10% for VEGF, 1% for MMP-2, 16% for MMP-3, 10% for MMP-7, 3% for MMP-9).

The laboratory staff was blinded with regard to the NPSLE status of patients during the analyses process, and SLE patient and healthy donor samples were randomized across assay layouts.

2.4 Statistics

Statistical calculations were carried out using Stata16 (StataCorp, College Station, TX). Numerical participant characteristics were reported as means with standard deviation (SD), while categorical characteristics were reported as counts and proportions. For comparisons of patient groups within each cohort, normality of variables was assessed using quantile-quantile plots. Approximation of MFAP4 and inflammatory biomarkers to normal distribution was achieved by $\log_{10}$ transformation before further statistical analysis. For comparisons between groups linear regression of log10-transformed measurements with Tukey's multiple testing correction was used. For SLICC and SLEDAI scores, groups were compared using Kruskal–Wallis test. Categorical data

were compared using Fisher's exact test. Seven IFN-α measurements were below limits of detection (LOD) and were ignored as missing data to allow usage of parametric tests. This in one way makes detection of significant changes in IFN-a levels difficult but is compensated by the increased power of parametric analyses. A p-value of <0.05 was used as limit of statistical significance. Pairwise associations between MFAP4, clinical variables, treatment status, cytokines or markers of BBB disruption were investigated using Spearman's correlation method or linear regression. We found no correlation (as an indication of degradation with long-term storage) between MFAP4 and time stored in freezer. When comparing NPSLE subgroups (CNS vs. PNS manifestations), the four patients with mixed CNS and PNS manifestations were excluded from analyses.

TABLE 1 Baseline demographics, clinical and treatment status of SLE cohort, NPSLE and non-NPSLE patients and healthy controls.

	SLE	Non-NPSLE[a]	NPSLE[a]	HCs	*p*-value
N		208	164	44	50
Sex					
Male	28 (13.5%)	24 (14.6%)	4 (9.1%)	8 (16.0%)	
Female	180 (86.5%)	140 (85.4%)	40 (90.9%)	42 (84.0%)	0.77
Age at time of diagnosis, mean (SD)					
	35.3 (14.9)	35.2 (14.6)	35.9 (16.2)		0.96
Age at time of blood sampling, mean (SD)					
	47.6 (14.4)	47.3 (14.9)	48.5 (12.3)	45.8 (13.9)	0.82
SLICC score, median (IQR) SLICC					
0–4	1 (0, 2)	1 (0, 2)	1 (0.5, 3)		0.063
>4	193 (93.2%)	156 (95.7%)	37 (84.1%)		
	14 (6.8%)	7 (4.3%)	7 (15.9%)		0.031
SLEDAI score, median (IQR) SLEDAI					
0–4	2 (0, 4)	2 (0, 4)	2 (2, 6)		0.013
5–8	193 (93.2%)	156 (95.7%)	37 (84.1%)		
>8	10 (4.8%)	4 (2.5%)	6 (13.6%)		0.057
	4 (1.9%)	3 (1.8%)	1 (2.3%)		
Plaquenil at time of blood samling					
No	100 (48.1%)	72 (43.9%)	28 (63.6%)		
Yes	100 (51.9%)	92 (56.1%)	16 (36.4%)		0.068
Prednisolon at time of blood sampling					
No	106 (51.0%)	86 (52.4%)	20 (45.5%)		
Yes	102 (49.0%)	78 (47.6%)	24 (54.5%)		0.72
Ever received immunotherapy[b]?					
No	15 (7.2%)	14 (8.5%)	1 (2.3%)		
Yes	193 (92.8%)	150 (91.5%)	43 (97.7%)		0.39
Treatment naive[c]					
No	204 (98.1%)	161 (98.2%)	43 (97.7%)		
Yes	4 (1.9%)	3 (1.8%)	1 (2.3%)		1.00

Count data were compared using Fisher's exact test. Normally distributed data (ages) were compared using ANOVA. Nonparametric data were compared using Kruskal–Wallis test.

SLE, systemic lupus erythematosus; non-NPSLE, non-neuropsychiatric systemic lupus erythematosus; NPSLE, neuropsychiatric systemic lupus erythematosus; HCs, healthy controls; SD, standard deviation; IQR, interquartile range.

[a]Non-NPSLE and NPSLE patients are subgroups within SLE cohort.

[b]Immunosuppressive therapy comprises: Azathioprin, Cyclophosphamide, Cytostatics and other DMARDs. Plaquenil is not considered immunosuppressive in this study.

[c]Treatment naive comprises patients who have not had treatment with immunosuppressive therapy or Plaquenil at any time after SLE diagnosis (ever) nor at the time of blood sampling (present).

3 Results

3.1 Baseline characteristics

Of 208 patients in the SLE cohort 44 patients (21%) were previously identified with NPSLE (24) while the rest (79%) are denoted as non-NPSLE in this study.

Only four patients (1.9%) were treatment naive at the time of blood sampling. Further baseline demographics, clinical characteristics, and treatment status of the total SLE cohort, NPSLE and non-NPSLE patients and HC are presented in Table 1.

3.2 Influence of age on MFAP4, GFAP and NfL

No difference was found between MFAP4 levels and sex within the total SLE cohort ($p = 0.87$) nor within the NPSLE group of patients ($p = 0.50$). Overall, a positive association was found between MFAP4 levels and patients' age at blood sampling ($\rho = 0.35$, $p < 0.001$). This positive association with age was also observed in healthy controls ($\rho = 0.30$, $p = 0.032$). When NPSLE/non-NPSLE was used as independent variables, this revealed an accelerated age-dependent increase in NPSLE ($\rho = 0.51$, $p < 0.001$) compared to non-NPSLE ($\rho = 0.31$, $p < 0.001$) ($p = 0.008$). This age-dependent increase in MFAP4 levels was even more prominent in CNS-group ($\rho = 0.59$, $p < 0.001$) compared to non-NPSLE ($\rho = 0.31$, $p < 0.001$) ($p = 0.014$).

A positive association was found between NfL levels and patients' age at blood sampling in healthy controls ($\rho = 0.75$, $p < 0.001$) as well as in the NPSLE ($\rho = 0.53$, $p < 0.001$) and non-NPSLE subgroups ($\rho = 0.36$, $p = 0.020$). When NPSLE/non-NPSLE was used as independent variables, this did not reveal an age-accelerated increase for NfL ($p = 0.962$).

A positive association was found between GFAP levels and patients' age at blood sampling in healthy controls ($\rho = 0.59$, $p < 0.001$) as well as NPSLE ($\rho = 0.46$, $p < 0.001$), however not within the non-NPSLE subgroup ($\rho = 0.25$, $p = 0.138$). When NPSLE/non-NPSLE was used as independent variables, this did not reveal an age-accelerated increase for GFAP ($p = 0.570$).

3.3 MFAP4 in NPSLE and non-NPSLE

MFAP4 levels were significantly elevated in patients with NPSLE compared to patients with non-NPSLE ($p = 0.031$), independent of age, more prominent in the NPSLE subgroup with CNS involvement ($p = 0.017$) (Figures 1A,B). No significant differences were found in MFAP4 between the total SLE cohort and HC ($p = 0.77$), nor between NPSLE ($p = 0.47$) and non-NPSLE compared to HC ($p = 0.73$).

3.4 Markers of neuronal and astrocyte injury

NfL and GPAP levels may be released into blood following CNS tissue damage. In this study NfL and GFAP were found to

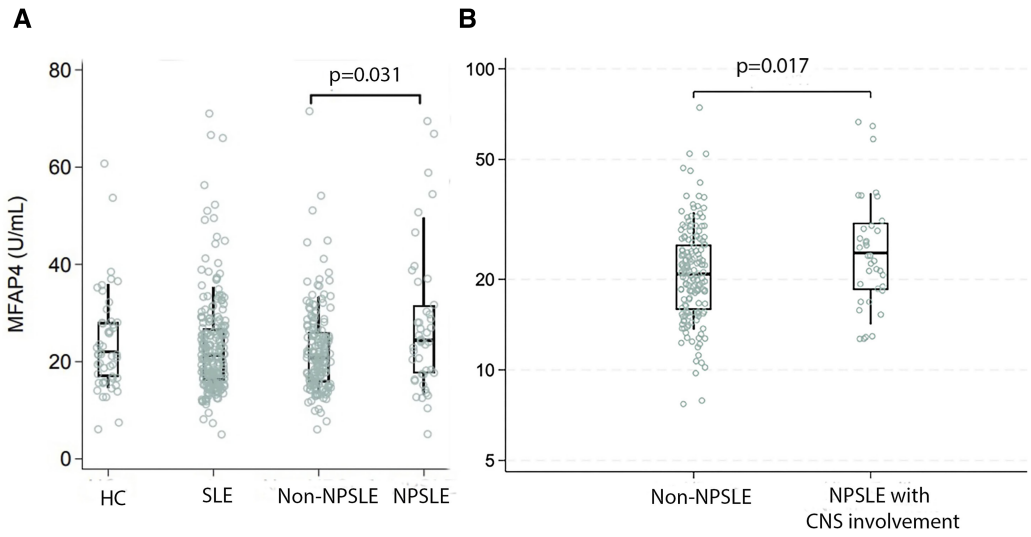

FIGURE 1
MFAP4 levels in total SLE cohort, non-NPSLE and NPSLE patients and healthy controls. Non-NPSLE and NPSLE patients are subgroups within the SLE cohort. MFAP4 was found significantly elevated in NPSLE (A) and more prominent in NPSLE patients with CNS involvement (B) compared to non-NPSLE patients. HC, healthy controls; SLE, systemic lupus erythematosus; NPSLE, neuropsychiatric systemic lupus erythematosus; Non-NPSLE, non-neuropsychiatric systemic lupus erythematosus; MFAP4, microfibrillar-associated protein 4.

be significantly higher in the total SLE cohort as well as in the NPSLE subgroup compared to HC (all $p < 0.001$) (Table 2). NfL and GFAP were found to be significantly higher in the NPSLE subgroup with CNS manifestations compared to HC ($p < 0.001$, $p < 0.001$) (Figures 2A,B). No significant difference was found between NPSLE and non-NPSLE (data not shown), neither

TABLE 2 Levels of MFAP4, cytokines, MMPs and VEGF in SLE, NPSLE, non-NPSLE and healthy controls.

Biomarkers sera	HCs	SLE vs. HCs	NPSLE vs. HCs	Non-NPSLE vs. HCs	NPSLE vs. non-NPSLE
MFAP4 (U/ml)	22.0 (17.0, 28.0)	21.3 (16.2, 26.7) ⇔	24.4 (17.6, 31.5) ⇔	20.8 (15.8, 26.0) ⇔	⇑ ($p = 0.031$)
IFN-α (pg/ml)	0.011 (0.006, 0.019)	0.066 (0.017, 0.501) ⇑ ($p < 0.0001$)	0.139 (0.018, 0.505) ⇑ $p < 0.0001$	0.060 (0.017, 0.476) ⇑ $p < 0.0001$	⇔
IL-6 (pg/ml)	0.34 (0.24, 0.73)	0.47 (0.31, 1.09) ⇑ ($p = 0.0099$)	0.66 (0.31, 1.34) ⇑ $p = 0.0026$	0.47 (0.31, 1.00) ⇑ $p = 0.030$	⇔
IL-10 (pg/ml)	0.15 (0.07, 0.31)	0.28 (0.15, 0.50) ⇑ ($p = 0.0009$)	0.28 (0.13, 0.47) ⇑ $p = 0.016$	0.28 (0.15, 0.50) ⇑ $p = 0.001$	⇔
IL-17 (pg/ml)	0.57 (0.40, 0.94)	0.73 (0.46, 1.57) ⇑ ($p = 0.042$)	0.98 (0.46, 2.00) ⇑ ($p = 0.025$)	0.69 (0.46, 1.57) ⇔	⇔
TNF-α (pg/ml)	0.73 (0.54, 1.07)	1.16 (0.70, 1.94)	1.19 (0.80, 2.12)	1.16 (0.69, 1.87)	⇔
MMP-2 (ng/ml)	26.6 (23.8, 30.0)	24.8 (21.6, 30.0) ⇔	25.0 (21.6, 29.1) ⇔	24.7 (21.4, 30.1) ⇔	⇔
MMP-3 (pg/ml)	1038.5 (714.8, 1333.4)	1390.1 (817.8, 2816.9) ⇑ (p$p = 0.0001$)	1340.9 (791.4, 2878.4) ⇑ $p = 0.003$	1400.9 (817.8, 2816.9) ⇑ $p = 0.002$	⇔
MMP-7 (pg/ml)	145.1 (93.4, 266.8)	212.0 (124.0, 436.7) ⇑ ($p = 0.0027$)	237.4 (126.7, 550.5) ⇑ $p = 0.010$	207.3 (122.0, 404.0) ⇑ $p = 0.048$	⇔
MMP-9 (ng/ml)	15.2 (11.7, 23.7)	17.0 (10.5, 26.5) ⇔	12.9 (8.0, 22.9) ⇔	17.8 (11.0, 28.7) ⇔	⇑ ($p = 0.048$)
VEGF (pg/ml)	10.1 (5.9, 16.2)	8.0 (4.5, 13.2) ⇔	6.8 (3.5, 10.5) ⇓ $p = 0.015$	8.1 (4.6, 13.5) ⇔	⇔
NfL (pg/ml)	7.4 (5.3, 10.1)	12.0 (7.7, 20.0) ⇑ $p < 0.0001$	13.0(8.7, 23.3) ⇑ $p < 0.0001$	11.9 (7.6, 19.8) ⇑ $p < 0.0001$	⇔
GFAP (pg/ml)	67.3 (47.5, 95.5)	105.348 (73.9, 160.4) ⇑ $p < 0.0001$	118.8 (78.2, 163.4) ⇑ $p < 0.0001$	102.7(73.487, 160.441) ⇑ $p < 0.0001$	⇔

Levels are expressed as median with interquartile range in brackets. Comparisons of groups are signified with a slash. For comparisons between groups linear regression of log10-transformed measurements with Tukey's multiple testing correction was used. ⇑ means elevated between groups compared, ⇓ means reduced between groups compared, and ⇔ means no difference between groups compared. NPSLE and non-NPSLE patients are subgroups within total SLE cohort, and therefore no comparisons were performed between these subgroups and the total SLE cohort.
SLE, systemic lupus erythematosus; NPSLE, neuropsychiatric systemic lupus erythematosus; Non-NPSLE, non-neuropsychiatric systemic lupus erythematosus; HCs, healthy controls; MFAP4, microfibrillar-associated protein 4; IFN-α, interferon alpha; IL, interleukin; TNF-α, tumor necrosis factor alpha; MMP, matrix metalloproteinase; VEGF, vascular endothelial growth factor.

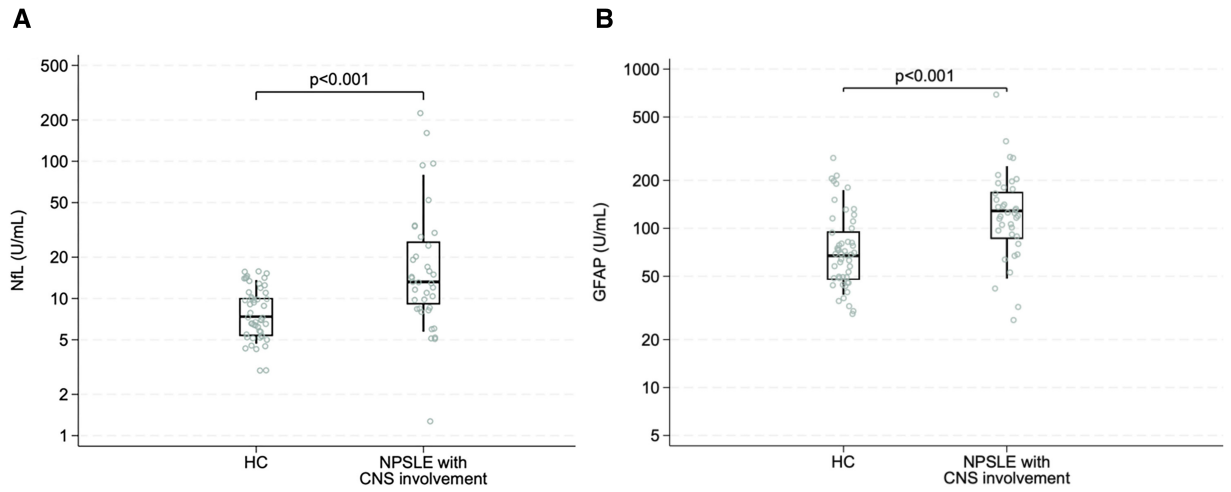

FIGURE 2
NfL and GFAP levels in NPSLE patients with CNS involvement compared to healthy controls. NfL (**A**) and GFAP (**B**) levels were significantly elevated in NPSLE patients with CNS involvement compared to healthy controls.

between the subgroup of NPSLE with CNS involvement and non-NPSLE (data not shown).

3.5 Markers of BBB disruption

Markers indicative of BBB disruption may serve as surrogate markers of NPSLE disease activity. Therefore, we examined MMPs (MMP-2, -3, -7, -9) and VEGF. A significant elevation in MMP-3 and MMP-7 levels was found in the total SLE cohort compared to HC ($p = 0.001$, $p = 0.0027$, respectively) and in NPSLE patients compared with HC ($p = 0.003$, $p = 0.010$). MMP-9 was elevated when comparing NPSLE and non-NPSLE ($p = 0.048$). Additionally, MMP-2 was significantly elevated in NPSLE patients with CNS manifestations compared to PNS manifestations ($p = 0.010$). VEGF was significantly reduced in NPSLE patients compared to HC ($p = 0.015$) (Table 2).

3.6 Markers of inflammation

We determined the serum levels of inflammatory markers, also presented in Table 2 for the NPSLE- and non-NPSLE-patients and HC. A significant elevation of IFN-α, IL-10, TNF-α, IL-6 and IL-17 was found in the total SLE cohort compared to HC ($p < 0.001$, $p = 0.0009$, $p = 0.0022$, $p = 0.0099$, $p = 0.042$, respectively). IFN-α, IL-10 and TNF-α were significantly elevated in NPSLE ($p < 0.001$, $p = 0.042$, $p = 0.007$, respectively) and in non-NPSLE compared to HC ($p < 0.001$, $p = 0.003$, $p = 0.004$). Also, a significant elevation in IL-6 and IL-17 was observed in the NPSLE compared to HC

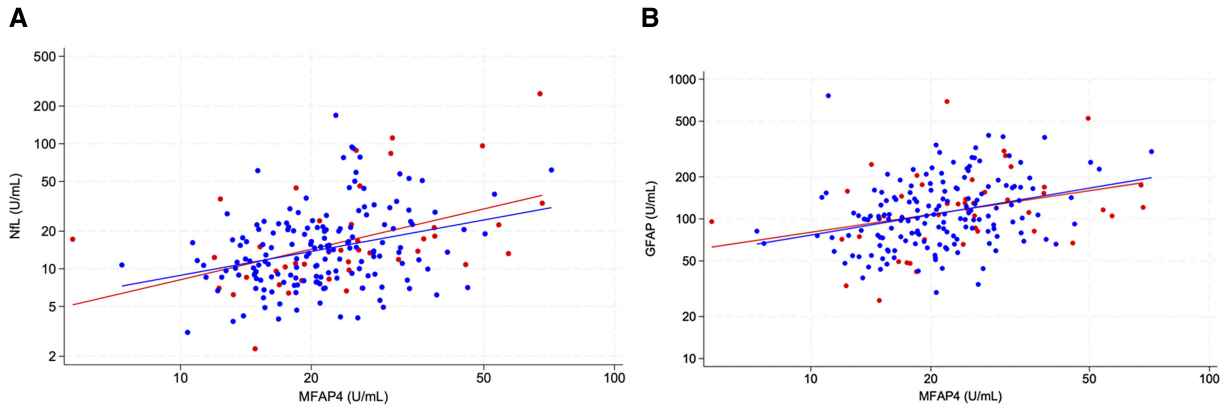

FIGURE 3
Correlation between NfL and MFAP4 (**A**) and GFAP and MFAP4 (**B**) in the NPSLE subgroup (red) and non-NPSLE subgroup (blue). A positive association was found between NfL and MFAP4 and GFAP and MFAP4 in the NPSLE as well as the non-NPSLE.

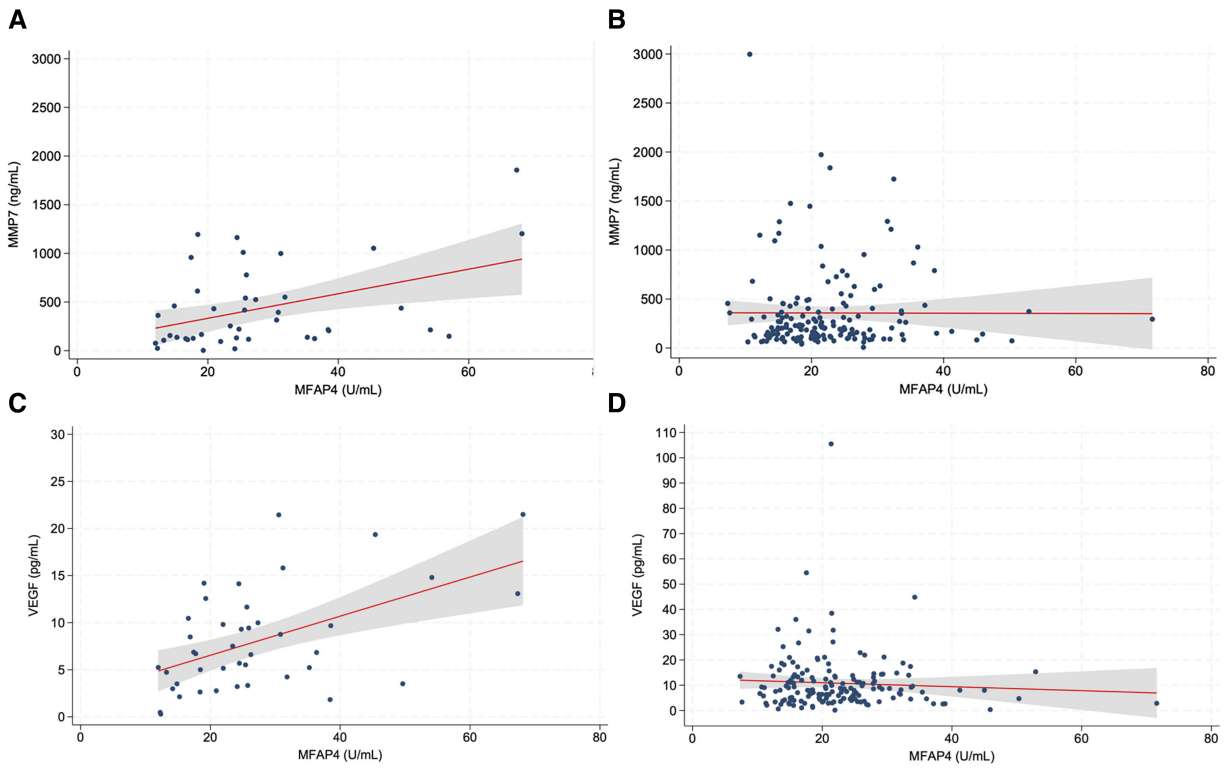

FIGURE 4
Correlation of MFAP4 with MMP-7 and VEGF in NPSLE subgroup and non-NPSLE subgroup. A significant association was found between MMP-7 and MFAP4 within NPSLE patients (**A**). Likewise, a significant association was found between VEGF and MFAP4 within NPSLE patients (**C**). No association was found between MMP-7 and MFAP4 (**B**) nor between VEGF and MFAP4 (**D**) in the non-NPSLE subgroup. Grey zone, confidence band. MFAP4, microfibrillar-associated protein 4; MMP-7, matrix metalloproteinase 7; VEGF, vascular endothelial growth factor; SLE, systemic lupus erythematosus; NPSLE, neuropsychiatric systemic lupus erythematosus.

($p = 0.0026$, $p = 0.025$), and for IL-6 also in non-NPSLE compared to HCs ($p = 0.030$).

3.7 Associations of MFAP4 and NfL, GFAP and markers of inflammation

Association between MFAP4, NfL and GFAP and inflammatory markers are shown in Supplementary Table S1.

In brief, a positive association was found between NfL and MFAP4 in the NPSLE as well as the non-NPSLE subgroup ($\rho = 0.44$, $p = 0.003$, $\rho = 0.25$, $p = 0.004$). This positive association with MFAP4 was also found for GFAP in the NPSLE as well as in non-NPSLE subgroup ($\rho = 0.33$, $p = 0.031$, $\rho = 0.31$, $p < 0.001$) (Figures 3A,B and Supplementary Table S1).

In the NPSLE-subgroup MMP-2 and MFAP4 was found to be significantantly associated ($\rho = 0.39$, $p = 0.014$), however this was not the case for MMP-9 ($\rho = -0.25$, $p = 0.256$). An association was found between MMP-7 and VEGF and MFAP4 in NPSLE patients ($\rho = 0.43$, $p = 0.023$, $\rho = 0.52$, $p < 0.001$, respectively) (Figure 4). No association was found between MMP-7 and VEGF and MFAP4 in non-NPSLE patients ($\rho = -0.003$, $p = 0.967$, $\rho = -0.062$, $p = 0.302$, respectively). An association was found between MMP-3 and VEGF and MFAP4 in NPSLE patients only with CNS manifestations ($\rho = 0.29$, $p = 0.023$, $\rho = 0.53$, $p = 0.001$, respectively), however not in the non-NPSLE subgroup ($\rho = -0.02$, $p = 0.836$).

Additionally, an association was found between IL-17 and MFAP4 and between TNF-α and MFAP4 in the NPSLE subgroup ($\rho = 0.43$, $p = 0.006$, $\rho = 0.36$, $p = 0.021$, respectively), which was not the case in the non-NPSLE subgroup ($\rho = -0.09$, $p = 0.245$, $\rho = -0.05$, $p = 0.507$, respectively (Figure 5).

3.8 Association between MFAP4 and clinical characteristics

A significant association between MFAP4 levels and SLICC score (accumulated disease damage) was found in the non-NPSLE subgroup ($\rho = 0.18$, $p = 0.003$). However, no association was found between MFAP4 levels and SLICC score when looking at the NPSLE subgroup ($p = 0.19$). No significant association was found between MFAP4 and SLEDAI score (present disease activity) neither in the total SLE cohort ($p = 0.83$) nor the NPSLE subgroup ($p = 0.89$).

4 Discussion

In this predominantly population-based cohort study of SLE with a longitudinal clinical follow-up, the main finding was that

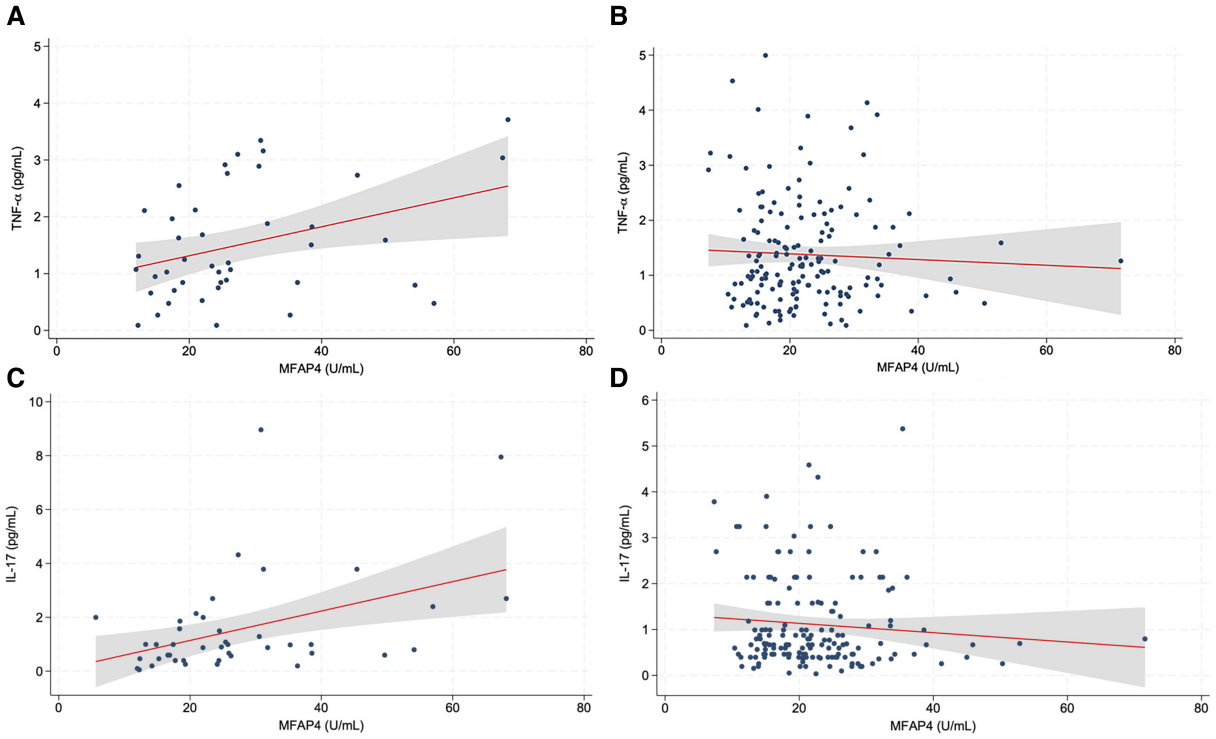

FIGURE 5
Correlation of MFAP4 with TNF-α and IL-17 in the NPSLE subgroup and non-NPSLE subgroup. A significant association was found between MFAP4 levels and TNF-α within the NPSLE subgroup (**A**). Furthermore, a significant association was found between MFAP4 levels and IL-17 within NPSLE subgroup (**C**). No association was found between TNF-α and MFAP4 (**B**) nor between IL-17 and MFAP4 (**D**) in the non-NPSLE subgroup. Grey zone, confidence band MFAP4, microfibrillar-associated protein 4; TNF-α, tumor necrosis factor alpha; IL-17, interleukin 17; SLE, systemic lupus erythematosus; NPSLE, neuropsychiatric systemic lupus erythematosus.

MFAP4 levels in the NPSLE subgroup were elevated compared to patients with non-NPSLE, more prominent in NPSLE patients with CNS involvement. In line with this observation markers of neuronal and astrocyte injury (NfL, GFAP) were significantly higher in the NPSLE subgroup with CNS manifestations compared to HC. A significant increase of inflammatory and BBB disruption markers was found in NPSLE subgroup. Remarkably, a positive correlation was found between MFAP4 levels and markers for neuronal (NfL) and astrocyte (GFAP) damage, BBB disruption (MMP-7 and VEGF) and inflammatory markers (TNF-α and IL-17). These data suggest that MFAP4, concurrent with MMPs and VEGF, is a marker for BBB disruption, and like cytokines may act as a marker for inflammation. A positive correlation between MFAP4 and NfL and GFAP may reflect tissue damage. These findings may contribute to the understanding of the pathogenesis of non-NPSLE as well as NPSLE.

The pathogenesis of NPSLE is complex and the precise mechanisms remain elusive (12, 35). BBB disruption is believed to be central in the pathogenesis of NPSLE (8, 13, 16). The BBB is a dynamic, non-absolute barrier that is highly regulated via the interactions between ECM proteins and ECM receptors such as integrin receptors (43). Thus, changes in the ECM components result in alterations of the functions of the BBB cells and leads to increased permeability of the BBB seen in pathological conditions of the CNS such as stroke, multiple sclerosis and Alzheimer's disease (12, 43). MFAP4 is an ECM protein that is located in the vascular ECM in most tissues (20, 21) and is measurable in serum (26). MFAP4 may directly affect the functions of the cells in the BBB as the primary cellular receptor for MFAP4 is integrin $\alpha_V\beta_3$ (44). Activation of vascular endothelial $\alpha_V\beta_3$ integrin results in disruption of VE-cadherin localized at the endothelial adherends junctions resulting in increased vascular permeability (43, 45). MMPs, one of the main proteolytic enzyme systems involved in ECM protein degradation during remodeling, is upregulated in pathological conditions such as stroke (43). MFAP4 has been linked with ECM remodeling during vascular injury (20, 44, 46, 47). We recently describe the expression of the MFAP4 in healthy human CNS, in connective tissue spaces of the brain including the meninges and the vascular/perivascular spaces. Notably, in autopsy samples from patients with acute multiple sclerosis (MS) and neuromyelitis optica spectrum disorder (NMOSD), we observed a downregulation of MFAP4 immunoreactivity at sites of active inflammation documented by the presence of inflammatory infiltrates. In line with these pathology data, cerebrospinal fluid (CSF) MFAP4 levels were reduced in active disease stages (27). The data suggested that inflammation changes the composition of ECM, perhaps due to the secretion of proteolytic enzymes e.g., MMPs in inflammatory conditions. In the present study, we

found MFAP4 to be upregulated in NPSLE patients compared to non-NPSLE patients. MMP-3 and MMP-7 were upregulated in NPSLE compared to HC. MMP-2 was upregulated in NPSLE patients with CNS manifestations compared to PNS manifestations. Furthermore, our data showed MFAP4 to be associated with markers of BBB disruption, MMP-7 and VEGF, in patients with NPSLE. MMP-3 and VEGF were found associated with MFAP4 within NPSLE patients only with CNS manifestations. This leads us to suggest that serum MFAP4 levels may reflect the BBB remodeling as a consequence of BBB disruption during CNS inflammation.

In this study higher levels of CNS-proteins, NfL and GFAP, were found in the total SLE cohort as well as NPSLE subgroup compared to HC. Notably, NfL and GFAP were significantly higher in the NPSLE subgroup with CNS manifestations compared to HC. This finding is in accordance with previous studies that reported that in NPSLE patients with CNS involvement, intrathecal levels of NFL and GFAP were increased (18, 19). Remarkably, in our study NfL and GFAP levels correlated positively with MFAP4, which may reflect CNS tissue damage.

Notably in NPSLE, IFN-α has been suggested to directly damage neurons and stimulate microglial engulfment of neurons after BBB disruption, and also to stimulate microglial production of proinflammatory cytokines, like IL-6 and IL-8 (13). IL-6 is believed to increase B-cell activation and survival (13) and may also play a role in BBB breakdown in NPSLE (14). IL-10 and TNF-α are also both thought to be regulators of the immune response in patients with NPSLE (13). IL-6 is furthermore considered to induce the production of IL-17 through T cell differentiation (48). IL-17 is a proinflammatory cytokine that plays a vital role in the pathogenesis of SLE (6, 48). In line with these events the present study shows that IFN-α, IL-6, IL-10, IL-17 and TNF-α were elevated in the total SLE cohort compared to HC. Furthermore, IFN-α, IL-6, IL-10 and TNF-α were significantly elevated in NPSLE compared to HC. Notably, IL-17 and MFAP4 were associated both in the total SLE cohort and in the NPSLE subgroup, and TNF-α was associated with MFAP4 in the NPSLE subgroup. Cytokines have been linked to clinical characteristics of SLE and NPSLE as cytokines drive the inflammatory cascade and lead to disease damage (1, 2, 8, 13, 48).

The study has several strengths. A large predominantly population-based cohort was used, including a significant number of patients with NPSLE. Secondly, analysis was done in a blinded fashion with detailed clinical information. A limitation of the study is the cross-sectional design. Furthermore, the relationship between MFAP4 levels and the type and degree of neuropsychiatric involvement cannot be established in this study due to the limited sample size. Experimental studies suggest that MFAP4 immune reactivity is increased in cardiovascular disorders, asthma and liver fibrosis (26, 49, 50). Moreover, in patients with asthma MFAP4 levels in serum were increased (50). SLE is a systemic autoimmune disease with various organ involvement. More data is required on the effects of different disease manifestations on MFAP4 levels. One may also argue that CSF reflects CNS pathology in a more direct and specific way compared to serum. We acknowledge the need to confirm the results in a prospective preferably multicenter study with longitudinal follow-up and with paired CSF and serum samples taken prior to treatment.

In conclusion, this study points to a potential role for MFAP4 in NPSLE additionally correlated with markers of BBB disruption and proinflammatory cytokines, suggesting that MFAP4 is a marker of inflammation and vascular re-modelling. Furthermore, data show that MFAP4 may act as a marker for tissue damage in CNS in line with NfL and GFAP in NPSLE with CNS involvement. These findings may contribute to the understanding of the pathogenesis of SLE as well as NPSLE. Further studies, preferably longitudinal, multicenter and with multiple ethnicities, on MFAP4 are warranted to validate the value of MFAP4 as a potential biomarker for NPSLE and SLE.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving humans were approved by the Regional Southern of Denmark. The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent for participation in this study was provided by the participants' legal guardians/next of kin.

Author contributions

MW: Investigation, Methodology, Formal Analysis, Data curation, Writing – original draft. SM: Data curation, Formal Analysis, Methodology, Software, Writing – review & editing. MO: Methodology, Formal Analysis, Writing – review & editing, Investigation. JM: Formal Analysis, Investigation, Writing – review & editing, Methodology. GS: Formal Analysis, Investigation, Methodology, Writing – review & editing, Data curation. AV: Data curation, Formal Analysis, Investigation, Methodology, Writing – review & editing, Software, Supervision. NA: Formal Analysis, Investigation, Methodology, Supervision, Writing – review & editing, Conceptualization, Funding acquisition, Validation.

Funding

The authors declare financial support was received for the research, authorship, and/or publication of this article.

This work was supported by the Danish Rheumatism Association (R193-A6886, R196-A6887).

Acknowledgments

We would like to thank for guidance and collaboration, Søren Lillevang for ensuring healthy controls for the study, Tine Drud Lüttge Rasmussen, Cancer and Inflammation Research at the Department of Molecular Medicine, University of Southern Denmark, and Dorte Aalund Olsen, Department of Biochemistry & Immunology, Vejle Hospital, for assistance in analysis of biomarkers.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The authors declared that they were an editorial board member of Frontiers, at the time of submission. This had no impact on the peer review process and the final decision.

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/flupu.2024.1386256/full#supplementary-material

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