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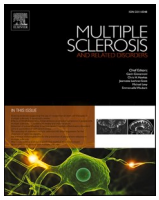

Blood-brain barrier damage associates with glia-related cytokines in the cerebrospinal fluid of patients with Multiple Sclerosis

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ABSTRACT

Background: The presence of Blood-Brain Barrier (BBB) dysfunction is defined by albumin quotient (Q_{ALB}) and characterize a group of Multiple Sclerosis (MS) patients at clinical onset. We evaluated the concentration in cerebrospinal fluid (CSF) of 87 cytokines, to better characterize the CSF inflammatory pattern in presence of BBB damage.

Materials and Method: In an exploratory cohort, CSF cytokines were evaluated by means of Multiplex technology (Bio-Plex Pro-Human Cytokine, GF and Diabetes 27-Plex Panel, Bio-Plex Pro-Human Chemokines 40-Plex Panel, Bio-Plex Pro-Human Inflammation Assays 37-Plex Panel) in a cohort of Other Not Inflammatory Neurological Disorders (ONIND) and in cohort of patients with MS, stratified according to BBB damage into Q^{+}_{ALB} and Q^{-}_{ALB} MS patients. In the validation cohort, we evaluated the relevant molecules in a cohort of MS patients, stratified again into Q^{+}_{ALB} and Q^{-}_{ALB}, including also Neurofilament Light (NfL) and Chitinase 3-like 1 (CHI3L1) CSF concentration.

Results: While MIP-1α, CXCL-13, and CCL-22 CSF concentrations were higher in both MS groups compared to ONIND, in Q^{+}_{ALB} MS CSF concentrations of CXCL-9 (17.85 ± 4.69 pg/mL), CXCL-10 (476.5 ± 324.3 pg/mL), and IL-16 (96.08 ± 86.17 pg/mL) were higher than in Q^{-}_{ALB} MS (8.98 ± 5368 pg/mL, $p < 0.005$, 281.0 ± 180.9 pg/mL, $p < 0.05$, and 47.35 ± 36.87 pg/mL, $p < 0.005$, respectively) and ONIND (8.98 ± 5368 pg/mlL, $p < 0.005$, 281.0 ± 180.9 pg/mL, $p < 0.005$, and 47.35 ± 36.87 pg/mL, $p < 0.001$, respectively). A strong correlation was observed between CXCL-9 and CXCL-10 in all MS groups (all $r>0.75$, all $p < 0.001$). In the validation cohort again CXCL-10 CSF concentration were higher in Q^{+}_{ALB} MS than in Q^{-}_{ALB} MS (94.25 ± 64.75 vs 153.8 ± 99.52, $p < 0.05$), while no difference was observed in serum. CSF NfL (1642 ± 1963 vs 3231 ± 3492 pg/mL, $p < 0.05$) and CHI3L1 (183.9 ± 86.62 vs 262 ± 137.5 ng/mL, $p < 0.05$) were increased in Q^{+}_{ALB} MS.

Conclusions: BBB damage in MS is linked to a specific CSF cytokines pattern (CXCL-9, CXCL-10, IL-16), that are also involved in astrocyte-microglia interaction. To what extent their continuous production in the CNS may mark a more severe disease course merits to be investigated.

1. Introduction

In Multiple Sclerosis (MS) the Blood-Brain Barrier (BBB) defines the border where immune system cells engage the Central Nervous System (CNS), thus playing a pivotal role in CNS autoimmune disorders (Sweeney et al., 2019; Spencer et al., 2018; Attfield et al., 2022). To estimate BBB integrity *in vivo*, the ratio between Cerebrospinal Fluid (CSF) and serum (S) Albumin (ALB) concentrations ($Q_{ALB}=ALB_{CSF}/ALB_{S}$ x 10^{-3}) has been applied (Puthenparampil et al., 2021; Puthenparampil et al., 2017; Puthenparampil et al., 2020; Lai et al., 2022; Musaeus et al., 2020). In Multiple Sclerosis (MS), BBB dysfunction (i.e., pathological Q_{ALB} values) has been recently linked to an increased cerebrospinal fluid (CSF) monocyte count, higher neurofilament light chain (NfL) and chitinase 3-like 1 (CHI3L1) levels, and different patterns of lymphocyte recruitment characterized by higher Effector Memory T-Helper cells (CD28- Th1) and low Th2. Puthenparampil et al. (2021) In addition, BBB

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Table 1
Clinical and Demographic variables. To compare these variable Chi square (a) and Mann Whitney (b) test applied. Abbreviations: ONIND, Other not inflammatory Neurological Disorders: pwMS: patients with Multiple Sclerosis; Q^{-}_{ALB}MS: pwMS without pathological Q_{ALB} values; Q^{+}_{ALB}MS: pwMS with pathological Q_{ALB} values.

	ONIND (16 patients)	pwMS (51 patients)	Q^{-}_{ALB}MS (43 patients)	Q^{+}_{ALB}MS (8 patients)	ONIND vs MS (p-values)	QALB+ vs QALB- (p-values)
Gender Ratio (F/M) [a]	2.2 (11/5)	2.9 (38/13)	3.8 (34/9)	1.0 (4/4)	0.76	0.18
Age at LP (y) [b] Mean±st.dev.	44.2 ± 8.0	35.7 ± 9.1	36.0 ± 9.5	34.1 ± 6.8	0.06	0.64
Disease duration at LP (m) [b] Mean±st.dev.	n.a.	13.3 ± 23.1	14.8 ± 24.7	5.4 ± 6.4	n.a.	0.45
EDSS [b] Median (range)	n.a.	1.5 (1.0–5.0)	1.5 (1.0–4.0)	1.5 (1.0–5.0)	n.a.	0.99
Clinical or radiological disease activity [a] n (%)	n.a.	23 (45 %)	18 (40 %)	6 (75 %)	n.a.	0.13

damage was associated with worse clinical and radiological prognosis. Uher et al. (2016) All these observations suggest that the presence of BBB damage in MS might mark a peculiar and more severe immunopathological process, an thus should be considered as independent variable for dissecting MS heterogeneity. To further characterized BBB dysfunction in patients with MS (pwMS), we evaluated whether it was associated with a specific pattern of CSF cytokines. Therefore, we studied 87 cytokines in the CSF of untreated patients with MS at very early disease phases and in patients with Other Neurological not Inflammatory Disorders (ONIND) and investigated their association with BBB dysfunction and clinical parameters.

2. Materials and methods

Study Populations. Two different cohorts of pwMS were included in the study. The *exploratory cohort* involved untreated (i.e., no steroids administrating in the last 28 days, no ongoing disease-modifying treatment) patients with relapse-onset MS at the time of the diagnosis and patients with Other Not Inflammatory Neurological Disorder (ONIND) constituted by individuals complaining tension headache, transient subjective sensory symptoms, and psychosomatic disorders, as well as unspecific white matter alterations, as previously described. Puthenparampil et al. (2020) Although no evidence of neurological or systemic diseases was achieved in these subjects, they were defined as ONIND rather than normal controls. The *validation cohort* involved untreated 80 RMS patients at the time of the diagnosis. MS diagnosis was achieved in agreement with the most recent diagnostic criteria. Thompson et al. (2017)

The study was approved by the Ethics Committee of the Azienda Ospedaliera di Padova (Prot. N. 17,760). A written informed consent was obtained from all patients and controls.

Serum and CSF analysis. Paired CSF and serum specimens were collected by nontraumatic lumbar puncture between 8.00 and 9.00 a.m.. Standard CSF examination included: cell count and differentiation, quantitative indexes of intrathecal IgG synthesis, detection of IgG oligiclonal bands, and BBB function (evaluated by means of the Q_{ALB}). BBB was defined for Q_{ALB} values (ALB_{CSF}/ALB_{Serum}) above the normal values (i.e., $Qlim_{Alb} = (\text{patient's age}/15)+4$) were considered pathological and expressed as $Q_{ALB\%}$ ($Q_{ALB}/ Qlim_{Alb}$), as already reported. Puthenparampil et al. (2016) CSF aliquots were stored at −80 °C until cytokines analysis.

CSF multiple cytokine investigation in the *exploratory cohort*. The concentrations of 87 cytokines were assessed in the CSF from the *exploratory cohort* by Multiplex technology (Bio-Plex Pro-Human Cytokine, GF and Diabetes 27-Plex Panel, Bio-Plex Pro-Human Chemokines 40-Plex Panel, Bio-Plex Pro-Human Inflammation Assays 37-Plex Panel). For each molecule the percentage of detectable concentration was evaluated. As already applied, cytokines detected in less than 50 % of all (MS and ONIND) samples were excluded from the analysis. Puthenparampil et al. (2020) When the same cytokine was detectable by two kits, results from the kit with higher sensitivity and frequency of detection were considered.

CXCL-10, NfL, and CHI3L1 ELISA in the *validation cohort*. CXCL-10 concentrations in paired serum and CSF samples from the *validation cohort* were evaluated by means of ELISA (Human CXCL10/IP-10 Immunoassay Quantikine® ELISA kit, Catalog Number DIP10, R&D System, Minneapolis), in line with manufacturer's instructions. In addition, NfL and CHI3L1 concentrations were quantified in CSF samples by ELISA (Human Diagnostics, Umea, Sweden, and MicroVue, Athens, OH, respectively) according to the manufacturer's instructions.

Statistical Analysis. Comparisons of CSF biomarkers concentration between multiple groups were explored with ANOVA or Kruskal–Wallis test as appropriate, with Tukey's or Dunn's correction respectively. A normal distribution test (Kolmogorov–Smirnov test) was performed to guide the choice of parametric or non-parametric test. Spearman Correlation analysis was performed to test the association between cytokines and Q_{ALB}%. A p-value lower than 0.05 was considered statistically significant. Prism 10.0.3 was used for all the analysis.

3. Results

Exploratory Cohort. In the exploratory cohort 51 RRMS patients and 16 ONIND were enrolled. Eight RRMS (out of 51, 15.7 %) and no ONIND had pathological Q_{ALB}. Based on this finding, MS cohort was divided into Q^{+}_{ALB} (8 patients) and Q^{-}_{ALB} (43 patients) MS patients. Demographic and clinical parameters are shown in Table 1. Complete CSF data are reported in Supplementary Table.

CSF cytokines could be classified as MS- or Q_{ALB}- related. MIP-1α, CXCL-13, and CCL-22 were linked to MS diagnosis. Indeed, their CSF concentrations were higher in both Q^{+}_{ALB} and Q^{-}_{ALB} MS patients compared to ONIND, with no difference between MS subgroups (Fig. 1A, Table 2). Moreover, no correlation with $Q_{ALB\%}$ was observed in both MS and ONIND (Fig. 1B).

On the other hand, CXCL-9, CXCL-10, and IL-16 were linked to BBB damage, since their CSF concentration was higher in Q^{+}_{ALB} MS compared to both Q^{-}_{ALB}MS and ONIND (Fig. 2A, Table 2). All of them correlated with $Q_{ALB\%}$ in MS, but not in ONIND (Fig. 2B).

Moderate to very high statistically significant correlations were observed between CXCL-9 and CXCL-10 in all groups (ONIND: r:0.62, $p = 0.012$; MS: r: 0.84, $p < 0.0001$; Q^{+}_{ALB} MS: r: 0.96, $p < 0.0001$; Q^{-}_{ALB} MS: r: 0.75, $p < 0.0001$).

In Validation Cohort CXCL-10 is increased in CSF but not serum. To confirm the relevance of CXCL-10 in RRMS, we included 80 patients (17 Q^{+}_{ALB}, and 63 Q^{-}_{ALB}) in a validation cohort, evaluating CXCL-10 concentrations in both CSF and serum with a different methodology (ELISA). A higher CSF concentration of CXCL-10 was confirmed in Q^{+}_{ALB} compared with Q^{-}_{ALB} (94.25 ± 64.75 vs 153.8 ± 99.52, $p = 0.0214$) also in this cohort, while serum concentrations did not differ (73.95 ± 178.0 in Q^{-}_{ALB} MS vs 53.41 ± 59.45 in Q^{+}_{ALB} MS, $p = 0.614$) (Supplementary Figure). No correlation was observed between CSF and serum

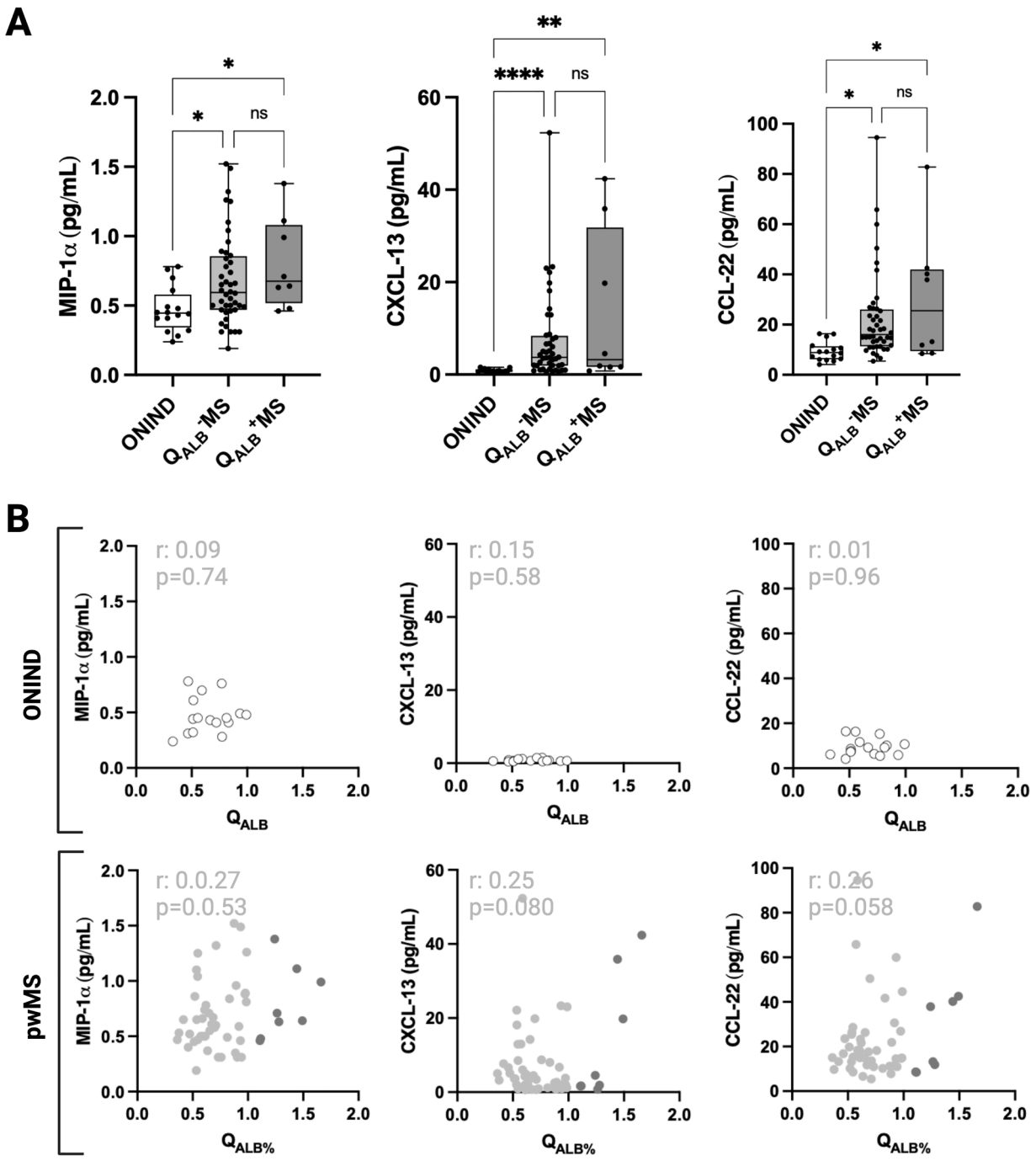

Fig. 1. MIP-1a, CXCL-13, CCL-22 increased in both MS groups. A) MIP-1a, CXCL-13, CCL-22 had increased CSF concentration in both Q^{-}_{ALB} ($n = 8$) and Q^{+}_{ALB} ($n = 43$) MS compared to ONIND ($n = 16$). Box plot: all values are plotted; box indicates 25–75 quartile. B) MIP-1a, CXCL-13, CCL-22 cytokines did not associate with $Q_{ALB\ \%}$ in both ONIND and MS patients. Values from Q^{-}_{ALB}MS are in light grey, while values from Q^{+}_{ALB}MS are in dark grey. Abbreviations: ONIND, Other not inflammatory Neurological Disorders: pwMS: patients with Multiple Sclerosis; Q^{-}_{ALB}MS: pwMS without pathological Q_{ALB} values; Q^{+}_{ALB}MS: pwMS with pathological Q_{ALB} values; * = $p < 0.05$; ** $p < 0.01$; ***; $p < 0.001$.

concentration (overall r: −0.02, $p = 0.898$; Q^{-}_{ALB} MS r: −0.06, $p = 0.646$; Q^{+}_{ALB} MS r: 0.19, $p = 0.945$). The significant correlation with $Q_{ALB\%}$ was confirmed (r: 0.38, $p < 0.001$) (Supplementary Figure) also in this cohort.

BBB dysfunction associates with higher CSF levels of NfL and CHI3L1.

To test previous findings in MS patients with BBB damage, we evaluated CSF concentration of NFL and CHI3L1. Compared to Q^{-}_{ALB}, Q^{+}_{ALB} MS had higher CSF concentration of both NfL (1642 ± 1963 vs 3231 ± 3492 pg/mL, $p = 0.019$) and CHI3L1 (183.9 ± 86.62 vs 262 ± 137.5 ng/mL, $p = 0.027$) (Fig. 3).

4. Discussion

Pathological Q_{ALB} values have been linked to different CSF cell patterns, higher CSF concentrations of NfL and CHI3L1, and increased intrathecal lipid species. Puthenparampil et al. (2021) To further characterize the immunopathological changes associated with BBB damage in RRMS, we initially evaluated 87 cytokines in the CSF of an exploratory cohort of RRMS at clinical onset and ONIND.

Table 2
MS- and BBB dysfunction- associated CSF cytokines. Values are expressed as mean ± standard deviation. Abbreviations: a: Kruskal-Wallis with Dunn's correction; b: Anova with Tukey's correction. Other abbreviation as in Table 1.

		ONIND			QALB$^-$MS			QALB$^+$MS			p-value	ONIND vs QALB$^-$ MS	ONIND vs QALB$^+$MS	QALB$^-$ vs QALB$^+$MS
MIP-1α	a	0.47	±	0.16	0.68	±	0.32	0.80	±	0.33	***0.009***	***0.025***	***0.021***	0.893
CXCL-13	a	0.80	±	0.35	7.28	±	9.47	13.56	±	17.03	***<0.0001***	***<0.0001***	***0.001***	>0.999
CCL-22	b	9.38	±	3.85	22.26	±	17.45	30.70	±	25.74	***0.007***	***0.020***	***0.013***	0.191
CXCL-9	b	6.55	±	4.67	8.98	±	5.37	17.85	±	14.69	***0.001***	0.450	***0.001***	***0.004***
CXCL-10	b	177.10	±	85.76	281.00	±	180.90	476.50	±	324.30	***0.002***	0.143	***0.001***	***0.022***
IL-16	b	27.36	±	14.82	47.35	±	36.87	96.08	±	86.17	***0.002***	0.238	***0.001***	***0.010***

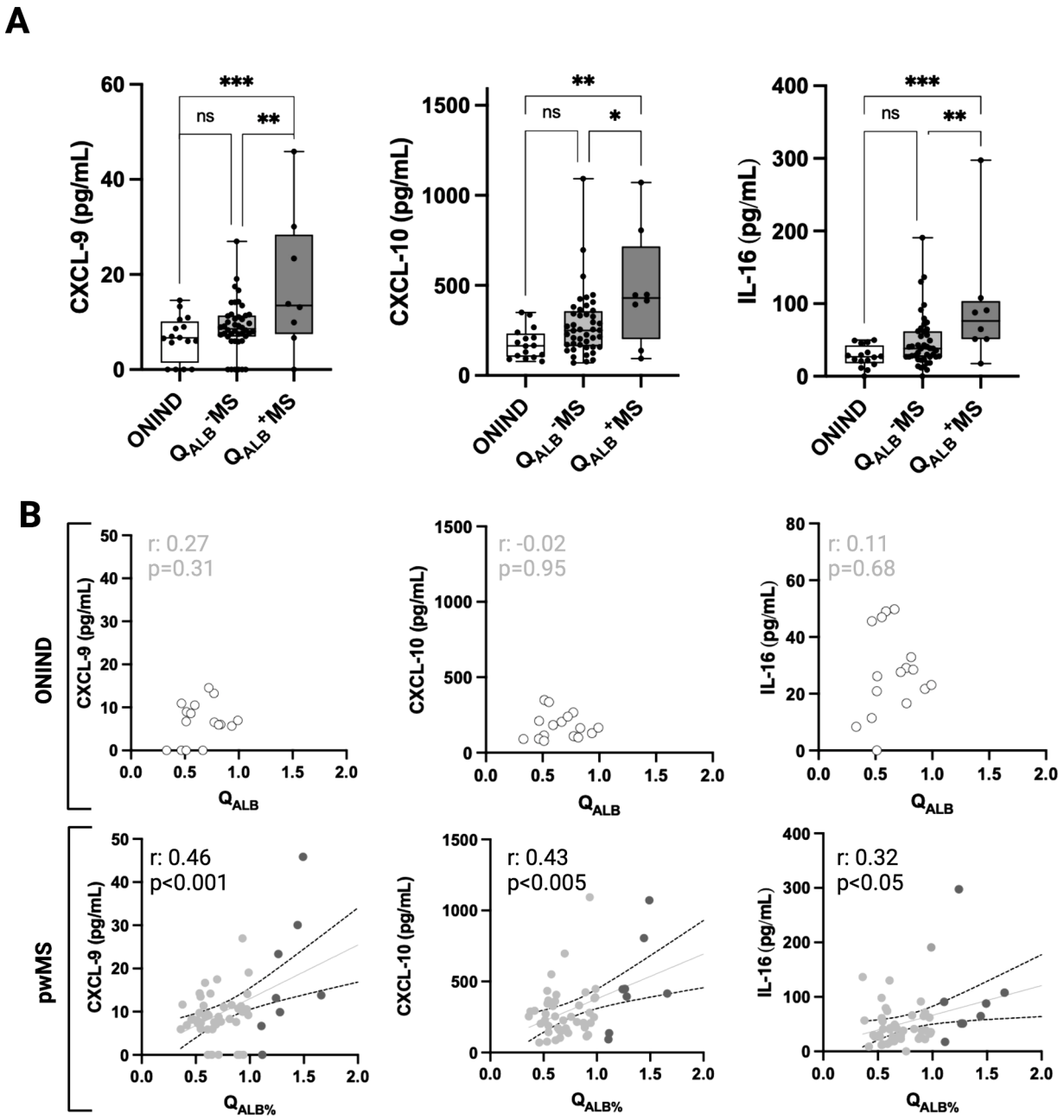

Fig. 2. CXCL-9, CXCL-10, and IL-16 increased in and Q^+_{ALB} MS. A) CXCL-9, CXCL-10, and IL-16 had increased CSF concentration in and Q^+_{ALB} MS ($n = 8$) compared to both ONIND ($n = 16$) and both Q^-_{ALB}MS ($n = 43$). Box plot: all values are plotted; box indicates 25–75 quartile. B) CXCL-9, CXCL-10, and IL-16 cytokines did not associate with $Q_{ALB\%}$ in ONIND, but in pwMS. Values from Q^-_{ALB}MS are in light grey, while values from Q^+_{ALB}MS are in dark grey. Abbreviations as in Fig. 1.

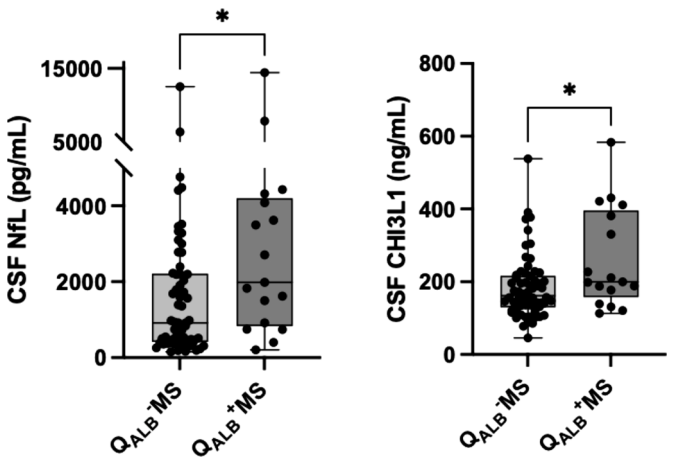

Fig. 3. In validation cohort Q_{ALB}^{+} MS had also increased NfL and CHI3L1 CSF concentrations. Abbreviations as in Fig. 1.

Three cytokines, namely MIP-1α, CXCL-13, and CCL-22, were increased in both Q_{ALB}^{+} and Q_{ALB}^{-} and, hence, were considered MS-specific. Increased CSF MIP-1α, previously pointed out in the literature, is a mark of glial activation, a histological feature of white matter inflammation in MS. Puthenparampil et al. (2020); Yi et al. (2014); Boven et al. (2000) This cytokine facilitates CNS penetration of autoreactive T-cells, as suggested by the increased expression of MIP-1α receptor (i.e., CCR5) on T-cells with the highest migratory rate. Zang et al. (2000); Von essen et al. (2023) A large amount of literature data confirms the increase of CXCL-13 in MS CSF. This cytokine was found to associate with both intrathecal IgGOB synthesis (Ferraro et al., 2015; Farina et al., 2017) and B- and T- lymphocyte recruitment (Kowarik et al., 2012; Krumbholz et al., 2006), with pathological MS findings of GM pathology (Farina et al., 2017) and with retinal microglia activation. Puthenparampil et al. (2022) Serum levels of both these two cytokines were normal in RMS, a finding strongly confirming their intrathecal synthesis. Puthenparampil et al. (2017); Melamud et al. (2022)

We found that CXCL-9, CXCL-10, and IL-16 were increased in both RRMS subgroups, but with a significant difference between Q_{ALB}^{+} and Q_{ALB}^{-}. The high CSF concentrations of CXCL-9 and CXCL-10 observed in our study are in line with previous reports *in vivo* (Iwanowski et al., 2017; Müller et al., 2010; Franciotta et al., 2001) and with a post-mortem study, where CXCL-10 was found in astrocytes around perivascular accumulations of $CXCR3^{+}$ lymphocytes and in active demyelinating plaques. Sorensen et al. (2002) Moreover, in human specimen from secondary progressive MS patients, astrocytes at the rim of the lesion were demonstrated to be the source of CXCL-10 in the CNS. Tanuma et al. (2006) In the same study, both local astrocytes and microglia were activated by this cytokine, in an autocrine and paracrine manner respectively. Taken all together, these findings might suggest that locally produced CXCL-10 by astrocytes might further activate microglia and induce BBB changes that facilitate the penetration of $CXCR\text{-}3^{+}$ cells in the context of CNS active demyelinating lesions.

The increased CSF IL-16 is in line with the observation of its expression in microglia and infiltrating T-cells, again suggesting an astrocyte-induced microglial activation. Hridi et al. (2021)

On the base of our findings, we may hypothesize that in RRMS the increased BBB permeability marks the presence of an increased macrophage/microglia and astrocyte activation, that might support both acute and chronic mechanisms of damage. Puthenparampil et al. (2021); Uher et al. (2016) Whether this dysfunction reflects primary astrocyte-microglial activation rather than an astrocyte-microglial activation induced by peripheral immunity is worth of further investigation.

Taking into consideration the converging literature on CXCL-10, we focused on this cytokine in the evaluation cohort and further confirmed its increase in the CSF in Q_{ALB}^{+}, with no difference in serum. Therefore, the correlation between Q_{ALB} and CXCL-10 CSF is determined by an increased intrathecal synthesis, and not by an increased passage from blood to CSF. Moreover, to verify our previous observations (Puthenparampil et al., 2021), we tested NfL and CHI3L1 CSF concentrations and confirmed that they were increased in presence of increased BBB permeability.

Our study presents three major limitations. First, we did not evaluate serum cytokine concentrations in the exploratory cohort, so we cannot evaluate if the BBB damage is induced by peripheral pro-inflammatory mediators or by primary microglial-astrocyte activation into the CNS. Second, the inclusion in the exploratory cohort of only 67 patients (16 ONIND and 51 RRMS) allowed us to find Q_{ALB}-associated cytokines, but we are aware that we could have not included others relevant. Finally, the absence of clinical follow-up calls for longitudinal studies, as we have already stated, to confirm the impact on brain atrophy and to evaluate whether other chronic aspects of MS immunopathogenesis associate with BBB dysfunction.

In summary, we demonstrated that the increased BBB permeability in RRMS is linked to specific CSF cytokines (CXCL-9, CXCL-10, IL-16). To what extent their continuous production in the CNS may mark a clinical more severe disease course merits to be further investigated.

Ethics approval and consent to participate

The study was approved by the 'Comitato Etico per la Sperimentazione Clinica dell'Azienda Ospedaliera di Padova' (Prot n 33n/AO/20), and all patients gave written informed consent.

Consent for publication

Not applicable.

Data availability

Anonymized data is available upon reasonable request.

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Authors' information

Not applicable.

CRediT authorship contribution statement

M Puthenparampil: Conceptualization, Formal analysis, Writing – original draft. **A Marin:** Formal analysis, Investigation. **G Zanotelli:** Formal analysis, Investigation. **VA Mauceri:** Formal analysis, Investigation. **F De Napoli:** Formal analysis, Investigation. **M Gaggiola:** Formal analysis, Investigation. **A Miscioscia:** Formal analysis, Investigation. **M Ponzano:** Formal analysis, Investigation. **F Bovis:** Formal analysis, Investigation. **P Perini:** Formal analysis, Investigation. **F Rinaldi:** Formal analysis, Investigation. **B Molon:** Formal analysis, Investigation. **P Gallo:** Conceptualization, Formal analysis.

Declaration of competing interest

M.P. reports grants from Almirall, Teva, Sanofi Genzyme, Merck Serono, Biogen Italy and Novartis; consultancy for Novartis, Biogen Italy and Sanofi Genzyme; board membership Sanofi Genzyme, Novartis and Biogen Italy. A.M., G.Z., V.M.A., F.D.N., M.G., A.M., M.Po. and B.M. have nothing to disclose. F.B. reports personal fees Biogen Italy, Roche, Avexis and Novartis. P.P. reports grants from Almirall, Teva, Sanofi Genzyme, Merck Serono, Biogen Italy, Novartis and Roche; consultancy

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.msard.2023.105403.

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