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Neurolymphatic biomarkers of brain endothelial inflammatory activation: Implications for multiple sclerosis diagnosis

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ABSTRACT

Aims: Multiple sclerosis (MS) is the leading cause of non-traumatic neurological disability in young adults, and its diagnosis is often delayed due to the lack of diagnostic markers. Initiation of disease -modifying therapy in the early stages of MS is especially critical because currently available therapy mostly target relapsing-remitting MS, and is less effective as disease progresses into the more chronic form of secondary-progressive MS. Therefore, exploring specific and sensitive biomarkers will facilitate an expedited and more accurate diagnosis to allow currently available therapies to be more effective.

Main methods: Western blotting was conducted to detect the expression of neurolymphatic proteins in human brain endothelial cells in culture. Additionally, using a cohort of 150 patients with relapsing remitting MS, 26 with secondary progressive MS, and 60 healthy control samples, neurolymphatic protein expression was detected in serum samples using dot blot analysis.

Key findings: Human brain microvascular endothelial cells express neurolymphatic markers. Neurolymphatic protein abundance increases with tumor necrosis factor (TNF)-α stimulation but decreases with interferon (IFN)-γ or combined (TNF + IFN) treatment. Circulating neurolymphatic protein levels is significantly lower in MS patients. Further, one of the markers, FOXC2, is associated with the clinical stages of MS, with significantly lower expression in secondary progressive MS compared to relapsing remitting MS.

Significance: Our findings describe brain endothelial expression of neurolymphatic proteins, which is altered under inflammatory stress, and provide a possibility of using a collective pool of circulating neurolymphatic proteins as a diagnostic and prognostic biomarker of MS.

1. Introduction

Multiple Sclerosis (MS) is a debilitating neurological disease characterized by inflammatory demyelination, neuronal degeneration and axonal loss [1]. MS affects > 850,000 people in the US [2] and 2.5 million worldwide [3], with most diagnoses made between the ages of 20–40 [4]. Most MS patients are initially diagnosed with the episodic 'relapsing-remitting' MS (RRMS) which often (75%) progresses to the chronic, 'progressive' form (secondary progressive MS (SPMS)) [5]. Currently available interventions limit MS 'flares' rather than slowing steady degeneration, leaving few treatment options once patients progress to SPMS. Thus, earlier diagnosis of MS may initiate treatments earlier, before degenerative progression sets in, allow currently available treatments to be more effective.

To date symptom based or stand−alone laboratory tests cannot accurately diagnose MS. The national MS society reports that MS diagnosis is 'often delayed, incorrect or even missed' [6]. Medical histories, neurologic exams and lab tests (including magnetic resonance imaging (MRI) and spinal fluid analysis) cumulatively allow physicians to rule out other neurological diseases, which confirm MS diagnoses. Behavioral tests for MS rely on the 'expanded disability status scale' (EDSS) that can also be subjective. Ultimately, the diagnosis (and

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initiation of treatment) is often only made *after* patients have sustained substantial central nervous system (CNS) damage which becomes visible as T2-enhancing lesions in MRI. Consequently, the importance of identifying other precise biomarkers of MS cannot be overstated.

In addition to current diagnostic methods, unbiased and mechanism-based testing methods are needed to improve and expedite MS diagnosis. This might be achieved by identifying additional groups of sensitive markers; the use of combined marker systems that are more highly powered would have improved statistical predictive power and lead to earlier disease discrimination. One such panel of MS markers are 'neurolymphatic' proteins, including prospero homeobox -1 (Prox-1), podoplanin, lymphatic vessel endothelial hyaluronan receptor-1 (LYVE-1), forkhead box c2 (FOXC-2), chicken ovalbumin upstream promoter-transcription factor ii (COUP-TFII) and vascular endothelial growth factor receptor-3 (VEGFR-3). We previously reported the 'ectopic' expression of these lymphatic endothelial-selective ('neurolymphatic') markers within the CNS in active experimental and human forms of multiple sclerosis (MS) [7] with the expression of these markers consistent with their endothelial origin, and spatial localization restricted to the vascular walls and perivasculature.

Therefore, although MS has long been viewed as an immune-mediated disease, vascular endothelial disturbances, including microparticle (MP) release and the potential role of the brain lymphatic system are increasingly receiving attention [8,9]. Brain endothelial cells constitutively release MPs, which are extracellular vesicles containing membrane and cytosolic components [10,11] and increase MP formation during endothelial stress. Because of their short half-lives, endothelial MPs ('EMP') provide a 'snapshot' of endothelial activation, reflecting the state of inflammatory and vascular stress [12]. So far, specific biomarkers that can be used to evaluate the formation of brain EMPs during active CNS inflammation have not been identified, but the validation of such markers could reveal EMP analysis as extremely useful in evaluating extent of cerebrovascular endothelial activation in MS, discriminating disease subtype, severity and responsiveness to therapy.

The objectives of our study were to demonstrate the reliability of the circulating neurolymphatic markers using a large collection of clinically defined blood samples from healthy human controls, and two types of MS samples.

2. Materials and methods

2.1. Clinical specimens

Clinical samples were obtained from University of Buffalo as previously described [13]. RRMS and SPMS patients and healthy controls (HC) were all enrolled at the Department of Neurology, University of Buffalo, Buffalo, NY. Inclusion criteria were: ages between 18 and 80 years, RRMS or SPMS [14] MS was diagnosed according to McDonald criteria [15], having had a MRI examination performed < 30 days of clinical examination with the standardized study protocol, and EDSS = 0–8.5. Exclusion criteria included relapse/exacerbation or steroid treatment < 30 days prior to study, having pre-existing conditions associated with non-MS brain pathology or pregnancy. HC subjects were recruited from hospital personnel and respondents to local advertisements. HC underwent physical examination, and were assessed for demographic characteristics, autoimmune and other concomitant diseases, vascular and environmental risks and personal habits. Samples of plasma were prepared by low speed centrifugation of acid-citrate dextrose anti-coagulated blood. Serum samples were prepared using serum collection tubes. All samples were stored at −80 °C until analysis at Louisiana State University Health Science Center-Shreveport (LSUHSC-S). The study was approved by Institutional Review Board at the University of Buffalo.

Table 1
Demographic data of healthy controls and multiple sclerosis patients.

	HC (n = 60)	MS (n = 177)	RRMS (n = 150	SPMS (n = 26)
Sex, female, n (%)	31 (52)	136 (77)	114 (76)	22 (85)
Age, years, mean (SD) median	44 (14) 44	46 (9.8) 47	45 (9.6) 45	55 (7) 54

HC: healthy controls; MS: multiple sclerosis; RRMS: relapsing–remitting; SPMS: secondary-progressive MS.

2.2. Isolation of microparticles

Serum and plasma samples were first centrifuged at 400 ×*g* for 10 min and the resulting supernatants were transferred to fresh microcentrifuge tubes and subjected to centrifugation at 20,800 ×*g* for 1 h at 4 °C to pellet MPs. Supernatants were aspirated and MP pellets were washed twice using 4 °C PBS plus 1 mM phenylmethylsulfonyl fluoride (PMSF) by centrifugation at 20,800 ×*g* for 15 min 4 °C. Supernatants were aspirated, and MP pellets stored at −80 °C until analyzed.

2.3. Cell culture

Human cerebral microvascular endothelial cells line (hCMEC/D3, provided by Dr. Pierre-Olivier Couraud, Inserm, France) was cultured in growth medium [EndoGRO™-MV complete media kit (Millipore, Burlington, MA)] on rat tail collagen type I (0.1 mg/mL) – coated flasks. Cells were kept at 37 °C in 5% CO2. Passages between 28 and 34 were used for all experiments.

2.4. Cytokine treatment

hCMEC/D3 cells were treated with growth medium supplemented with tumor necrosis factor (TNF)-α (20 ng/mL, Thermo Fisher Scientific), interferon (IFN)-γ (1000 U/mL, Thermo Fisher Scientific) or both 24 h after the cells reached confluency.

2.5. Western blotting

Western blotting was performed on serum and plasma specimens as well as hCMEC/D3 brain endothelial cell samples. To prepare whole cell and MP lysates from D3 cells cultured in six-well transwell plates, media were removed from the top and bottom chambers and MPs were isolated as described. Cells were washed with phosphate-buffered saline (PBS) and lysed in Laemmli buffer (Biorad, Hercules, CA). Cell lysates were then harvested by cell scraping, lysates were sonicated for 15 s (sec), boiled and frozen at −80 °C. Western blotting of serum and plasma samples were performed as previously described [7]. Briefly, five microliters of thawed specimens were loaded and separated by electrophoresis. Membranes were immunoblotted for prospero homeobox -1 (Prox-1), podoplanin, lymphatic vessel endothelial hyaluronan receptor-1 (LYVE-1), forkhead box c2 (FOXC-2) or vascular endothelial growth factor receptor-3 (VEGFR-3) and normalized against actin band intensity.

2.6. Dot blot analysis

Serum samples were diluted 1:1 with PBS and 0.5 μl was loaded onto nitrocellulose membrane and allowed to dry completely overnight. Dried membranes were blocked in 5% bovine serum albumin (BSA) in tris-buffered saline (TBS) -Tween prior to Ponceau S staining for protein loading identification. Membranes were immunoblotted for Prox-1, podoplanin, LYVE-1, chicken ovalbumin upstream promoter-transcription factor ii (COUP-TFII) or FOXC-2 and scan intensity was used for analyses.

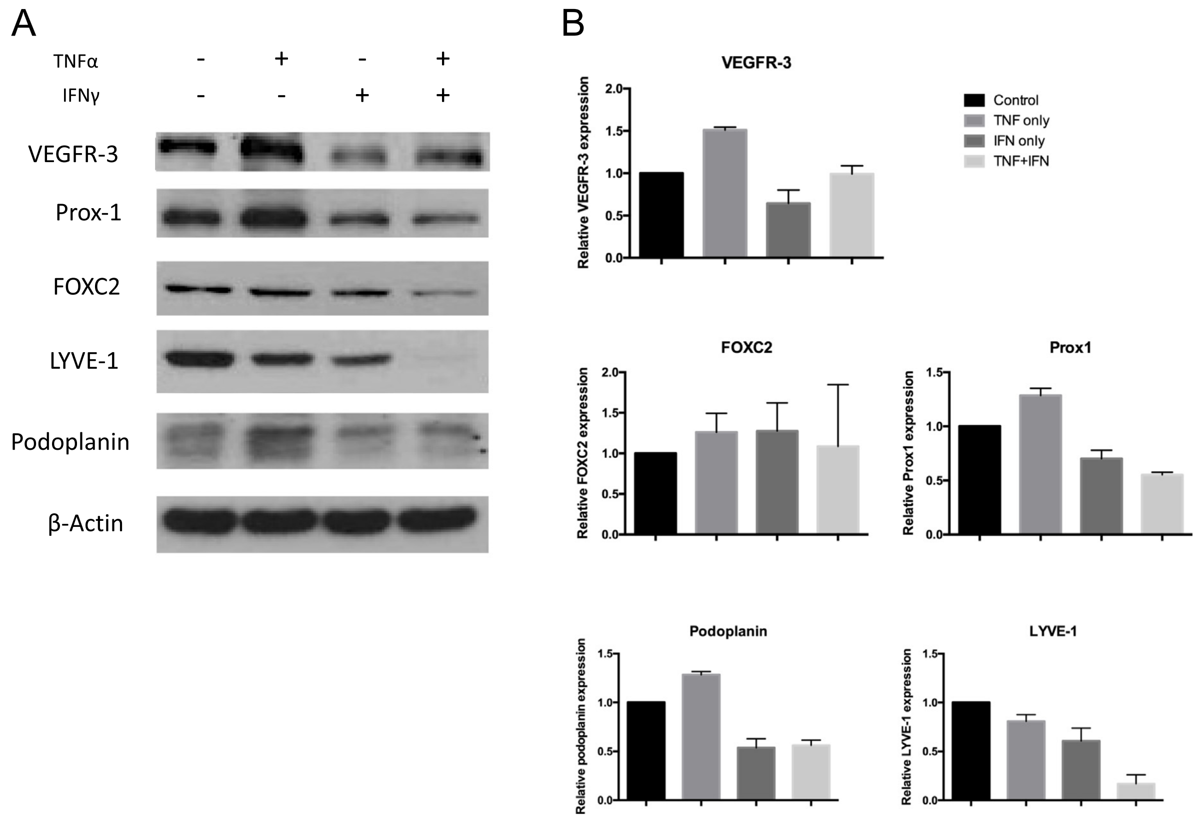

Fig. 1. Western blots of neurolymphatic proteins in hCMEC/D3 human brain microvascular endothelial cells. (A) Representative bands from western blots of hCMEC/D3 cell samples. Two-way ANOVA with unpaired *t*-test. (B) Quantification of western blots. *n* = 3 for each group. *p < 0.05, **p < 0.01, ***p < 0.001. One-way ANOVA with Dunnett post-hoc test was used.

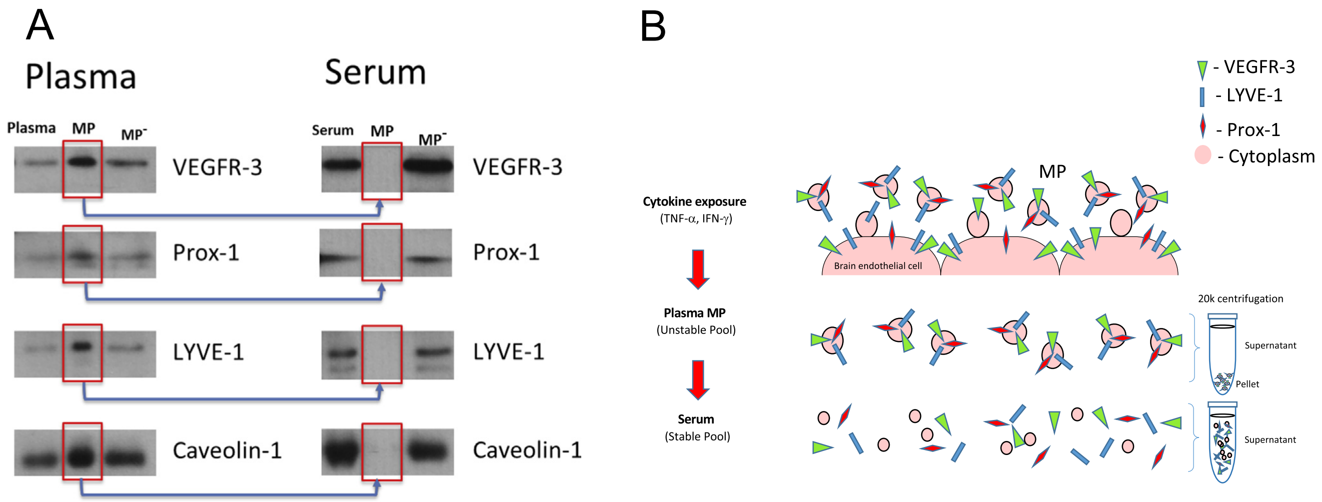

Fig. 2. Release and progressive conversion of brain endothelial microparticle-associated neurolymphatic markers. (A) Neurolymphatic proteins are largely restricted to the MP fraction in the plasma pool, which can be centrifuged; it is important to note that use of plasma microparticles could lead to varying sample recovery depending on the time of centrifugation after collection, as the MPs in the sample are undergoing progressive fission. Since serum samples have already undergone MP fission process, they represent a much more stable pool for analysis. '*Plasma*' represents unfractionated plasma; '*Serum*' represents unfractionated serum; *MP* specifies MP fraction isolated by 20,000 ×*g* centrifugation, MP^{-} denotes supernatant fraction after MP isolation. (B) Schematic representation of the fate of neurolymphatic markers released from brain endothelial cells. Brain endothelial cells release MPs containing neurolymphatic markers into the circulation. In plasma samples these MPs can be centrifuged to obtain a concentrated analyte that can be studied. However, in serum samples neurolymphatic protein-containing MPs have undergone progressive fission, liberating these markers and therefore it is no longer possible to centrifuge out the markers in MP pellets.

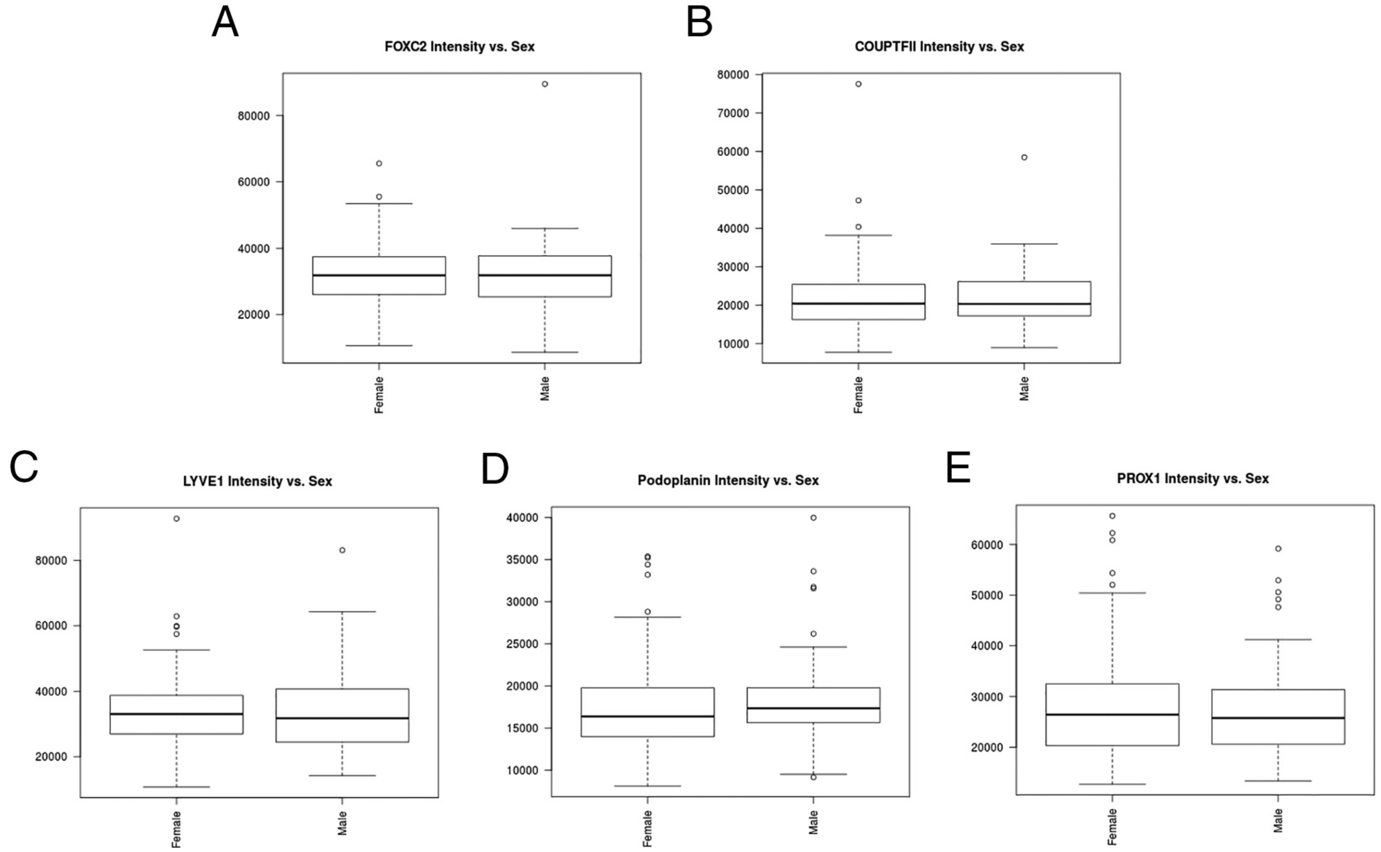

Fig. 3. Box plots showing the distribution of neurolymphatic protein expression levels of the serum samples grouped by sex. Serum samples analyzed included 60 of HC, 150 of RRMS and 26 of SPMS. Box plot explanation: upper horizontal line of box, 75th percentile; lower horizontal line of box, 25th percentile; horizontal bar within box, median; upper horizontal bar outside box, 90th percentile; lower horizontal bar outside box, 10th percentile. Student *t*-test was performed *$p < 0.05$ **$p < 0.01$ ***$p < 0.001$.

2.7. Antibodies

Prox-1 (cat no. ab37128, Abcam, Cambridge, MA), LYVE-1 (cat no. ab14917, Abcam), FOXC2 (cat no. ab65141, Abcam), VEGFR-3 (cat no. ab27278, Abcam), actin (cat no. A2103, Sigma-Aldrich, St. Louis, MO) were used for immunoblotting.

2.8. Statistical analysis

Basic statistical analysis was carried out using Student's unpaired, two-sided *t*-test to compare the HC cohort to the RRMS and SPMS cohorts for each protein in the data. The distribution of each protein was characterized using box-and-whisker diagrams. Association between proteins was characterized using one-way analysis of variance (ANOVA).

A heatmap was produced to show global relationships between all of the samples in the data. The row dendrogram is constructed using a modified average-linkage clustering algorithm which preserves group association; clustering occurs in such a way that all samples within a single cohort are clustered, while the interfaces between cohorts use the records with the least distance between them. Additionally, one-dimensional heatmaps ("heat-strips") were created to depict the distribution of each protein in isolation.

Statistical analyses (GraphPad Software, Inc., La Jolla, CA) were performed using one-way ANOVA with Dunnett post-hoc test to compare neurolymphatic marker expression in brain endothelial cells in culture. Two-tailed Student *t*-tests were used to compare clinical samples between groups. A p value < 0.05 was considered statistically significant.

3. Results

3.1. Demographic features of participants

One hundred and fifty patients with RRMS, twenty-six with SPMS, and sixty healthy controls (HC) participated in the study. Study subjects with MS had a mean age of 46 years (± SD = 9.8, median 47). The mean age of HC subjects was 44 ± 14 (SD) years (median = 44). 45 MS patients were female and 15 were male. These demographic characteristics are presented in Table 1.

3.2. Brain endothelial cells express lymphatic endothelial markers

Previously, we reported the *ectopic* expression of lymphatic endothelial specific markers (including podoplanin and LYVE-1) in postmortem MS brain specimens associated with the vasculature. This time, we demonstrated that brain microvascular endothelial cells expression lymphatic specific protein, which here we refer to as "neurolymphatic" proteins. hCMEC/D3 cells indeed express VEGFR-3, Prox-1, FOXC2, LYVE-1 and podoplanin. The abundance of these proteins was altered in response to inflammatory cytokine stimulation. In particular, we observed a general trend of increased abundance following treatment with tumor necrosis factor (TNF)-α stimulation alone, and a significant decrease following interferon (IFN)-γ or combined stimulation (Fig. 1).

3.3. MP-associated markers are progressively liberated into the supernatant (soluble) fraction

Following exposure to inflammatory cytokines, brain endothelial cells transfer neurolymphatic proteins into microparticles (MPs), which

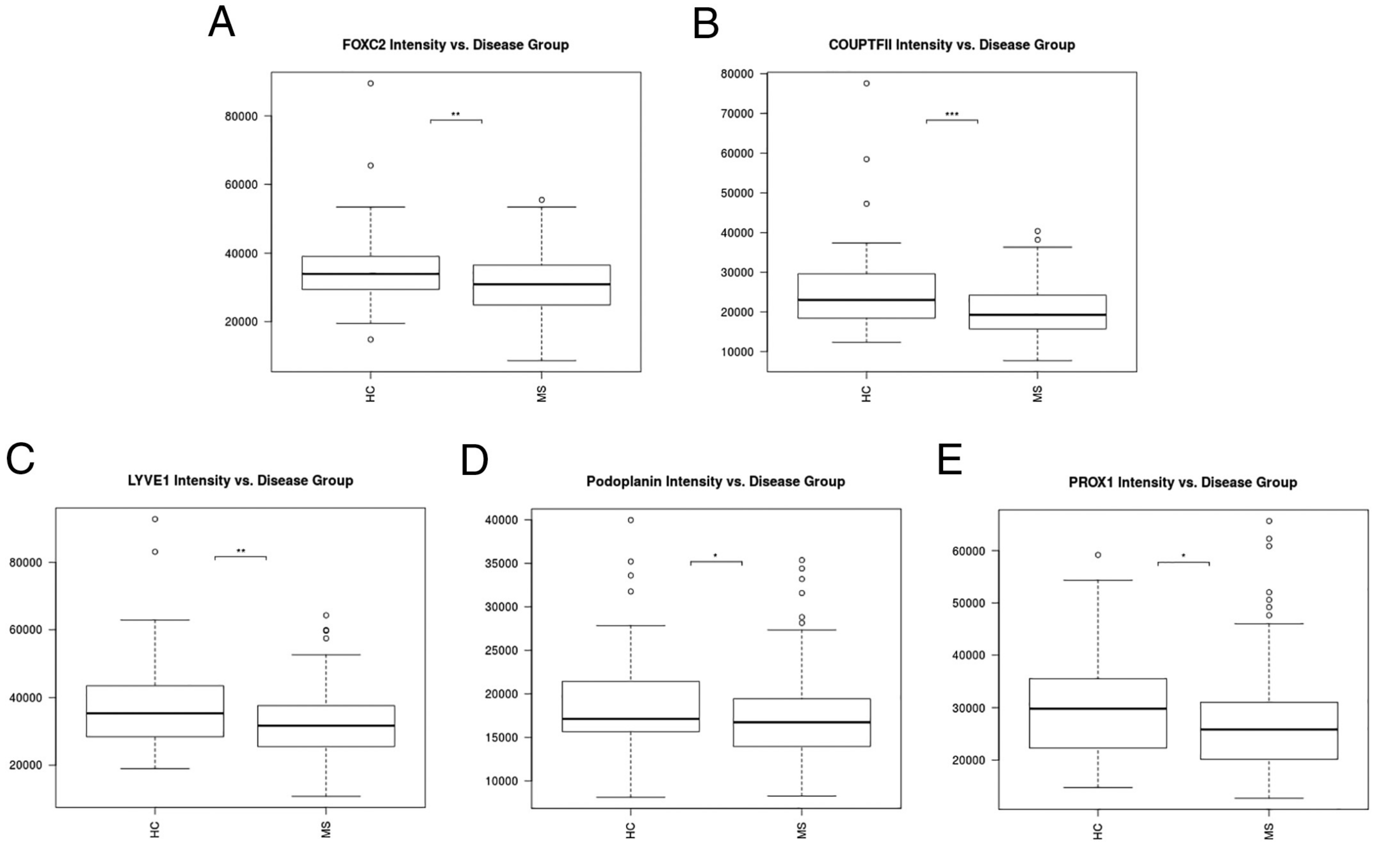

Fig. 4. Box plots depicting the distribution of serum neurolymphatic protein levels, grouped by the presence of disease. Expression levels are significantly lower in MS for FOXC2 (A), COUP-TFII (B), LYVE-1 (C), podoplanin (D) and prox1 (E) compared to HC. Student *t*-test was performed to compare each protein between HC and MS. *$p < 0.05$ **$p < 0.01$ ***$p < 0.001$.

are shed into the plasma (in vivo) and into the culture supernatant (in vitro). These MPs can be isolated by high-speed centrifugation. Following storage, retention of lymphatic biomarkers within MPs decreases, releasing these biomarkers into the supernatant phase. When we compared serum versus plasma samples, serum samples represented a more stable pool of these markers compared with the progressively fragmenting plasma samples. Thus, the MP associated lymphatic biomarkers can be relatively more reliably evaluated in the serum pool since this is the final compartment for these markers. (Fig. 2). Therefore, the following data presented here utilized serum samples.

3.4. Circulating neurolymphatic exhibit no sex differences

Because MS occurs much more commonly in women than men [16] we first evaluated possible sex-dependent differences in circulating neurolymphatic markers between men and women. No significant differences in the levels of FOXC2, COUP-TFII, LYVE-1, podoplanin and Prox1 with respect to sex in the analysis of serum samples (Fig. 3).

3.5. Lymphatic proteins can discriminate MS patients from healthy controls

We next investigated whether our panel of lymphatic markers could significantly discriminate MS patient serum samples (RRMS and SPMS) from HC. We found that circulating lymphatic protein levels were significantly lower in MS samples for all biomarkers (Fig. 4) and the averages for each neurolymphatic biomarker in MS were found to be reduced compared with HC; FoxC2 reduced in MS vs. HC (**$p < 0.01$), COUPTF II reduced in MS vs HC (***$p < 0.001$), LYVE1 reduced in MS vs HC (**$p < 0.01$), podoplanin reduced in MS vs HC (*$p < 0.1$) and Prox1 reduced in MS vs HC (*$p < 0.05$).

3.6. Lymphatic markers discriminate MS subtypes

Next, we investigated whether serum lymphatic markers could further discriminate MS subtypes (RRMS and SPMS) from HC. We found that while FoxC2, COUP-TFII, LYVE1, podoplanin and Prox1 could each differentiate HC from RRMS (*$p < 0.05$, ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$, *$p < 0.05$, respectively vs. HC), only FoxC2, COUP-TFII and LYVE-1 each showed significant differences between SPMS and HC (***$p < 0.001$, **$p < 0.01$, *$p < 0.05$, respectively vs. HC) (Fig. 5). FOXC2 was significantly different in SPMS compared with HC (***$p < 0.001$) and could additionally discriminate between subtypes of RRMS and SPMS (*$p < 0.05$) (Fig. 5A). All data from box plots are summarized in Table 2.

3.7. MS is associated with decreased circulating neurolymphatic markers

Heat map analyses allowed for effective visualization of the overall differences in the expression pattern of neurolymphatic proteins between the three groups: HC, RRMS and SPMS (Fig. 6). Each column of this heat map represents one lymphatic marker, from FoxC2 on the left through COUP-TFII, LYVE1, podoplanin to Prox1. Each row represents one sample (60 HC samples, 150 RRMS and 26 SPMS samples) with the level of expression mapped to the color scale of red to green. In general, these heat map patterns of each protein appeared to show differences among proteins, with FOXC2 showing the highest (bright green) level of expression while COUPTF-II was lower than other proteins, especially in HC and RRMS samples.

4. Discussion

MS is a devastating disease of the central nervous system (CNS)

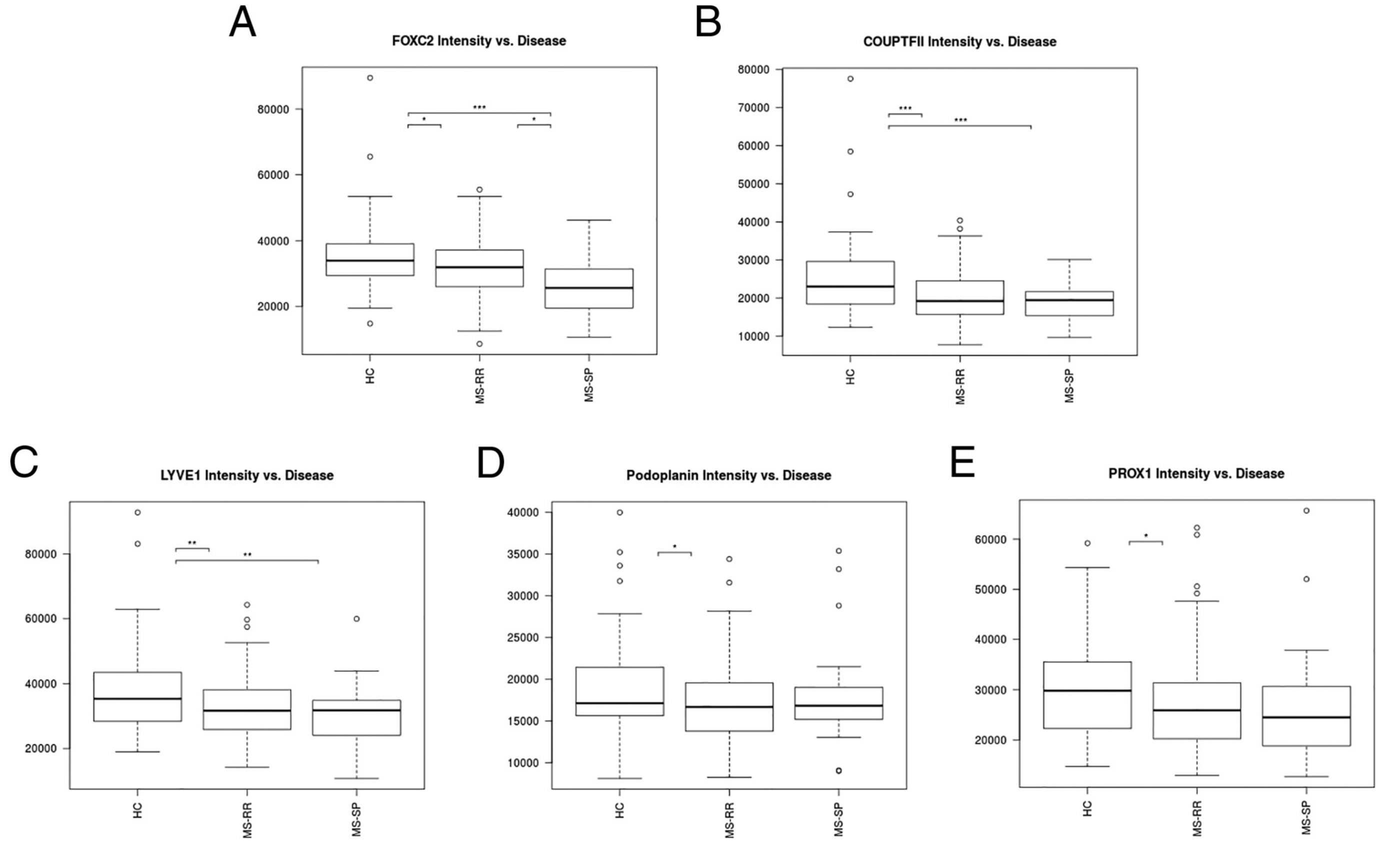

Fig. 5. Box plots showing the distribution of neurolymphatic protein expression levels of the serum samples grouped by MS disease type. Protein levels were significantly lower in RRMS compared to HC for COUP-TFII (B), LYVE-1 (C), podoplanin (D) and prox1 (E). Serum neurolymphatic levels were significantly lower in SPMS for FOXC2 (A), COUP-TFII (B), and LYVE-1 (C). $*p < 0.05$ $**p < 0.01$ $***p < 0.001$.

Table 2
Serum neurolymphatic levels altered with MS disease.

Mean intensity	HC (n = 60)	MS (n = 176)	RRMS (n = 150)	SPMS (n = 26)
Prox1		↓, p = 0.0298 vs. HC	↓, p = 0.030 vs. HC	↓, p = 0.20 (n.s. vs. HC)
COUP-TFII		↓, p = 0.00035 vs. HC	↓, p = 0.00055 vs. HC	↓, p = 0.00037 vs. HC
FOXC2		↓, p = 0.010 vs. HC	↓, p = 0.0427 vs. HC	↓, p = 0.00015 vs. HC ↓, p = 0.0042 SP vs. RR
LYVE-1		↓, p = 0.0058 vs. HC	↓, p = 0.010 vs. HC	↓, p = 0.0094 vs. HC
Podoplanin		↓, p = 0.02 vs. HC	↓, p = 0.0128 vs. HC	p = 0.46 (n.s. vs. HC)

Summary of the changes in neurolymphatic protein expression in serum samples. HC: healthy controls; MS: multiple sclerosis; RRMS: relapsing–remitting; SPMS: secondary-progressive MS.

which may represent a cytokine mediated neuroinflammatory state which drives demyelination and neurodegeneration. However, in addition to these neurological features, vascular inflammation and endothelial disturbances, in particular, have been increasingly noted in MS, which may also contribute to the pathophysiology of this condition. Indeed, hCMEC/D3 brain endothelial cells in culture were found to express several lymphatic endothelial markers including Prox-1, FOXC2, LYVE-1, podoplanin, VEGFR-3 and the COUP-TF-II.

Prox-1 is a transcription factor that is specifically induced by COUP-TFII that leads to differentiation of venous endothelial cells (VEC) into LECs during embryonic development [17]. Prox-1 activates the expression of lymphatic-specific proteins [18,19]. One such protein is podoplanin, which is one of the most important regulator of the separation of the cardiovascular and lymphatic systems [20]. VEGFR-3 is also upregulated by prox-1 and establishes a positive feedback loop with prox-1 to maintain LEC identity [21]. LYVE-1 is a hyaluronan receptor that is most frequently used as a LEC marker due to their expression being almost entirely restricted to LECs [22]. Lastly, FOXC2 is critical for maturation of lymphatic plexus into collecting vessels as well as lymphatic valve formation [23,24]. Although the functions of these lymphatic proteins in the brain endothelial cells are yet to be determined they regardless serve as sensitive markers of brain endothelial activation in response to inflammatory cytokines.

The abundance of these biomarkers was also significantly altered by exposure of human brain microvascular endothelial cells to inflammatory cytokines. Specifically, we found that there was an average

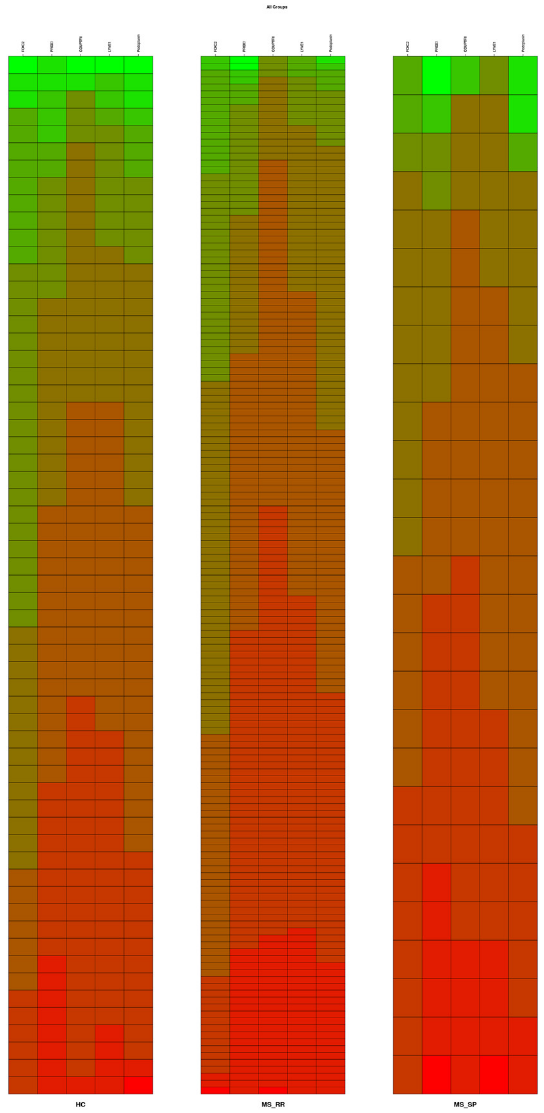

Fig. 6. Combined heat strip analyses depicting the distribution of each protein by disease states. The value in the heat strips is scan intensity of the dot blots. Individual proteins are arranged in columns, from left to right: FOXC2, Prox1, COUP-TFII, LYVE-1 and podoplanin. Rows represent individual samples. Within each column, samples are arranged from the highest (green) to lowest (red) expression. There are 60 samples for HC, 150 samples for RRMS and 26 samples for SPMS which explains the differences in to sizes and the number of rows for each group. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

1.5-fold increase in neurolymphatic protein levels in response to TNF-α stimulation alone. This is consistent with several reports of increased lymphatic expansion associated with inflammation, such as in inflammatory bowel disease and [25] myocarditis [26,27].

Interestingly, however, following stimulation with IFN-γ, we observed a *downregulation* of *all* neurolymphatic proteins in hCMEC/D3 cells. These results are similar to findings published by Kataru et al., which showed that T lymphocytes negatively regulate lymphatic expansion, both during steady and inflammatory states, via secretion of IFN-γ that suppresses lymphatic-specific gene expression in lymphatic endothelial cells (LEC)s [28]. In addition, accumulating evidence supports inflammation-associated lymphatic expansion to be not only a phenomenon that results from inflammation but a dynamic process, which involves both pro-lymphangiogenic and anti-lymphatic molecules, that shapes the course of inflammation [29]. This may also explain why we observed a downregulation of neurolymphatic proteins with a combined cytokine treatment.

Endothelial cells normally release MPs and increase their release under inflammatory conditions [8,30]. EMPs therefore may mirror the activation state of the endothelium, and provide a sampling of the vascular surface explaining their emergence as selective biomarkers of endothelial dysfunction [31]. Development of reliable panel of markers that are 1) expressed by the brain endothelium 2) altered in response to the state of activation e.g. that occurs which during MS and 3) transferred to MPs and be sampled from the circulation would potentially provide a useful tool for MS diagnosis as well as disease monitoring. The release of MPs results in the transfer of proteins from brain endothelial cells into a circulating pool, which can be analyzed as a ‘liquid biopsy’ of the endothelial surface to provide information on the state of activation and inflammation within the brain. Thus, we investigated whether circulating MPs express neurolymphatic markers and found that plasma samples contained neurolymphatic proteins and it was possible to concentrate the expression by isolation of plasma MPs (Fig. 3A).

However, upon further analyses of serum samples, we discovered that markers which are originally largely restricted to MPs become liberated into the soluble, non-centrifugable fraction, which in fact simplifies their analysis. Consequently, while we have identified MPs as a transitional intermediate location for neurolymphatic markers, the continuous fission of MPs into ‘microsomes’ seems to transfer these markers into a stable serum, but not plasma, samples which does not require centrifugation to obtain. Therefore, it may ultimately be simpler (and more reliable) for assays based on these sample to either employ non-centrifuged or frozen-stored serum samples. While it is theoretically possible that an immediate centrifugal isolation step to concentrate MPs from serum could yield an even more concentrated analyte, our studies so far indicate this may not be necessary.

Because MS predominantly affects women [32,33], we investigated the possible sex differences in neurolymphatic marker expression but found no significant sex-based differences. This finding may indicate that the neurolymphatic markers studied here are likely not necessarily related to sex-based differences seen in forms of MS. Also, in line with our in vitro results (obtained from hCMEC/D3 cells) that showed a downregulation of neurolymphatic proteins with combination treatment using TNF-α + IFN-γ stimulation, (which recapitulates the ‘cytokine storm; of MS [34]), we observed a significantly *lower* level of circulating markers with MS disease compared with HC (Fig. 4). When the MS group was further divided into subtypes (RRMS and SPMS) we found that different protein markers were sensitive to different groups, which allowed for more fine-tuned discrimination of samples. COUP-TFII, LYVE-1, podoplanin and Prox1 were each found to be different between RRMS and HC. Additionally, COUP-TFII and LYVE-1 showed significant differences between SPMS compared to HC. FOXC2 appeared to be the only marker that was able to differentiate between the subtypes of MS. Further characterization of additional protein biomarkers will be necessary for finetuning this type of sample discrimination.

In this study, we did not confirm the levels of IFN-γ or TNF-α in our serum samples. Similarly, we did not know what phase of disease or disease duration existed at the time of the sample collections. We also were not able to control for therapies being administered to these patients. Even so, with these limitations, we found several interesting differences among these groups which may support the use of these markers as a useful panel for studying MS diagnosis, staging and response to therapy. Future studies which might collect samples from patients during active MS flares and in treatment naïve-patient patients might yield more robust findings to validate these biomarkers. Such additional, unbiased and mechanism-based testing methods are still necessary for MS diagnosis in order to expedite earlier and more accurate MS diagnosis.

We have demonstrated that lymphatic proteins expressed by the brain microvascular endothelial cells reflect the state of activation utilization appear to be liberated into the circulation that can be sampled. Additional sensitive markers are still needed to develop a more reliable biomarker matrix. The sample required in these tests is a small (< 50 uL) blood/serum sample which could be frozen-stored, shipped to a test facility without requiring immediate access to clinical laboratories conducting western blot analyses. This approach could be accomplished using samples which are already being collected and could lower costs, reduce needs for patient travel and allow for more frequent testing at reduced costs. Such samples could be obtained on demand, analyzed using relatively inexpensive supplies and stored frozen until analyzed in clinical laboratories. The correlation of these markers with other validated markers like MRI and EDSS might demonstrate to which extent this testing might be applicable in the evaluation of MS disease activity.

5. Conclusions

Brain microvascular endothelial cells express and release neurolymphatic proteins into MPs which can be captured by centrifugation, demonstrating the presence of lymphatic proteins in MPs. The association of neurolymphatic markers with MPs is lost through MP fission, liberating these tracers from MPs in serum. The levels of these proteins detected in the circulation were all found to be significantly lower in MS patients, with several different proteins in the panel of markers tested here showing differences between MS patient samples and HC. Although intriguing, these biomarkers require additional several steps of validation but may ultimately be applied as a tool in MS diagnosis and disease monitoring.

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References

[1] M.T. Wallin, W.J. Culpepper, J.D. Campbell, L.M. Nelson, A. Langer-Gould, R.A. Marrie, et al., The prevalence of MS in the United States: a population-based estimate using health claims data, Neurology 92 (10) (2019) (e1029-e40).

[2] S.V. Ramagopalan, A.D. Sadovnick, Epidemiology of multiple sclerosis, Neurol. Clin. 29 (2) (2011) 207–217.

[3] Organization WH, Multiple Sclerosis International Federation. Atlas of Multiple Sclerosis, (2015).

[4] De Judicibus MA, McCabe MP. The impact of the financial costs of multiple sclerosis on quality of life. International journal of behavioral medicine. 2007;14(1):3–11.

[5] T. Vollmer, The natural history of relapses in multiple sclerosis, J. Neurol. Sci. 256 (Suppl. 1) (2007) S5–13.

[6] National MS Society, Diagnosing MS, Available from: https://www.nationalmssociety.org/Symptoms-Diagnosis/Diagnosing-MS.

[7] G.V. Chaitanya, S. Omura, F. Sato, N.E. Martinez, A. Minagar, M. Ramanathan, et al., Inflammation induces neuro-lymphatic protein expression in multiple sclerosis brain neurovasculature, J. Neuroinflammation 10 (2013) 125.

[8] J.W. Yun, A. Xiao, I. Tsunoda, A. Minagar, J.S. Alexander, From trash to treasure: the untapped potential of endothelial microparticles in neurovascular diseases, Pathophysiology: the official journal of the International Society for Pathophysiology/ISP 23 (4) (2016) 265–274.

[9] J.W. Yun, A. Minagar, of the Nervous A-JS, Emerging roles of endothelial cells in multiple sclerosis pathophysiology and therapy, Inflammatory Disorders of the Nervous, 2017.

[10] S. Hosseinzadeh, M. Zahmatkesh, M.R. Zarrindast, G.R. Hassanzadeh, M. Karimian, A. Sarrafnejad, Elevated CSF and plasma microparticles in a rat model of streptozotocin-induced cognitive impairment, Behav. Brain Res. 256 (2013) 503–511.

[11] A. Minagar, W. Jy, J.J. Jimenez, W.A. Sheremata, L.M. Mauro, W.W. Mao, et al., Elevated plasma endothelial microparticles in multiple sclerosis, Neurology 56 (10) (2001) 1319–1324.

[12] A. Rank, R. Nieuwland, A. Crispin, S. Grützner, M. Iberer, B. Toth, et al., Clearance of platelet microparticles in vivo, Platelets 22 (2) (2011) 111–116.

[13] J.S. Alexander, R. Chervenak, B. Weinstock-Guttman, I. Tsunoda, M. Ramanathan, N. Martinez, et al., Blood circulating microparticle species in relapsing-remitting and secondary progressive multiple sclerosis. A case-control, cross sectional study with conventional MRI and advanced iron content imaging outcomes, J. Neurol. Sci. 355 (1–2) (2015) 84–89.

[14] F.D. Lublin, S.C. Reingold, Defining the clinical course of multiple sclerosis: results of an international survey. National Multiple Sclerosis Society (USA) Advisory Committee on Clinical Trials of New Agents in Multiple Sclerosis, Neurology 46 (4) (1996) 907–911.

[15] C.H. Polman, S.C. Reingold, B. Banwell, M. Clanet, J.A. Cohen, M. Filippi, et al., Diagnostic criteria for multiple sclerosis: 2010 revisions to the McDonald criteria, Ann. Neurol. 69 (2) (2011) 292–302.

[16] H.F. Harbo, R. Gold, M. Tintoré, Sex and gender issues in multiple sclerosis, Ther. Adv. Neurol. Disord. 6 (4) (2013) 237–248.

[17] J.T. Wigle, N. Harvey, M. Detmar, I. Lagutina, G. Grosveld, M.D. Gunn, et al., An essential role for Prox1 in the induction of the lymphatic endothelial cell phenotype, EMBO J. 21 (7) (2002) 1505–1513.

[18] K. Mishima, T. Watabe, A. Saito, Y. Yoshimatsu, N. Imaizumi, S. Masui, et al., Prox1 induces lymphatic endothelial differentiation via integrin alpha9 and other signaling cascades, Mol. Biol. Cell 18 (4) (2007) 1421–1429.

[19] Y.-K.K. Hong, N. Harvey, Y.-H.H. Noh, V. Schacht, S. Hirakawa, M. Detmar, et al., Prox1 is a master control gene in the program specifying lymphatic endothelial cell fate, Developmental dynamics: an official publication of the American Association of Anatomists 225 (3) (2002) 351–357.

[20] G. Oliver, K. Alitalo, The lymphatic vasculature: recent progress and paradigms, Annu. Rev. Cell Dev. Biol. 21 (2005) 457–483.

[21] R.S. Srinivasan, N. Escobedo, Y. Yang, A. Interiano, M.E. Dillard, D. Finkelstein, et al., The Prox1-Vegfr3 feedback loop maintains the identity and the number of lymphatic endothelial cell progenitors, Genes Dev. 28 (19) (2014) 2175–2187.

[22] S. Banerji, J. Ni, S.X. Wang, S. Clasper, J. Su, R. Tammi, et al., LYVE-1, a new homologue of the CD44 glycoprotein, is a lymph-specific receptor for hyaluronan, J. Cell Biol. 144 (4) (1999) 789–801.

[23] A. Sabine, Y. Agalarov, H. Maby-El Hajjami, M. Jaquet, R. Hägerling, C. Pollmann, et al., Mechanotransduction, PROX1, and FOXC2 cooperate to control connexin37 and calcineurin during lymphatic-valve formation, Dev. Cell 22 (2) (2012) 430–445.

[24] T.V. Petrova, T. Karpanen, C. Norrmen, R. Mellor, T. Tamakoshi, D. Finegold, et al., Defective valves and abnormal mural cell recruitment underlie lymphatic vascular failure in lymphedema distichiasis, Nat. Med. 10 (9) (2004) 974–981.

[25] F. Becker, P. Yi, M. Al-Kofahi, V.C. Ganta, J. Morris, J.S. Alexander, Lymphatic dysregulation in intestinal inflammation: new insights into inflammatory bowel disease pathomechanisms, Lymphology 47 (1) (2014) 3–27.

[26] M. Al-Kofahi, J.W. Yun, A. Minagar, J.S. Alexander, Anatomy and roles of lymphatics in inflammatory diseases, Clinical and Experimental Neuroimmunology 8 (3) (2017) 199–214.

[27] I. Kholová, G. Dragneva, P. Cermáková, S. Laidinen, N. Kaskenpää, T. Hazes, et al., Lymphatic vasculature is increased in heart valves, ischaemic and inflamed hearts and in cholesterol-rich and calcified atherosclerotic lesions, Eur. J. Clin. Investig. 41 (5) (2011) 487–497.

[28] R.P. Kataru, H. Kim, C. Jang, D.K. Choi, B.I. Koh, M. Kim, et al., T lymphocytes negatively regulate lymph node lymphatic vessel formation, Immunity 34 (1) (2011) 96–107.

[29] J. Paupert, N.E. Sounni, A. Noël, Lymphangiogenesis in post-natal tissue remodeling: lymphatic endothelial cell connection with its environment, Mol. Asp. Med. 32 (2) (2011) 146–158.

[30] D. Burger, A.C. Montezano, N. Nishigaki, Y. He, A. Carter, R.M. Touyz, Endothelial microparticle formation by angiotensin II is mediated via Ang II receptor type I/NADPH oxidase/Rho kinase pathways targeted to lipid rafts, Arterioscler. Thromb. Vasc. Biol. 31 (8) (2011) 1898–1907.

[31] P.J. Yong, C.H. Koh, W.S. Shim, Endothelial microparticles: missing link in endothelial dysfunction? Eur. J. Prev. Cardiol. 20 (3) (2013) 496–512 (journal of WSN).

[32] M. Debouverie, S. Pittion-Vouyovitch, S. Louis, F. Guillemin, L. Group, Natural history of multiple sclerosis in a population-based cohort, Eur. J. Neurol. 15 (9) (2008) 916–921.

[33] A. Alonso, M.A. Hernán, Temporal trends in the incidence of multiple sclerosis: a systematic review, Neurology 71 (2) (2008) 129–135.

[34] J. Philippé, J. Debruyne, G. Leroux-Roels, A. Willems, J. Dereuck, In vitro TNF-alpha, IL-2 and IFN-gamma production as markers of relapses in multiple sclerosis, Clin. Neurol. Neurosurg. 98 (4) (1996) 286–290.