

# Lipocalin-2 is increased in progressive multiple sclerosis and inhibits remyelination

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## ABSTRACT

**Objective:** We aimed to examine the regulation of lipocalin-2 (LCN2) in multiple sclerosis (MS) and its potential functional relevance with regard to myelination and neurodegeneration.

**Methods:** We determined LCN2 levels in 3 different studies: (1) in CSF and plasma from a case-control study comparing patients with MS (n = 147) with controls (n = 50) and patients with relapsing-remitting MS (n = 75) with patients with progressive MS (n = 72); (2) in CSF and brain tissue microdialysates from a case series of 7 patients with progressive MS; and (3) in CSF at baseline and 60 weeks after natalizumab treatment in a cohort study of 17 patients with progressive MS. Correlation to neurofilament light, a marker of neuroaxonal injury, was tested. The effect of LCN2 on myelination and neurodegeneration was studied in a rat in vitro neuroglial cell coculture model.

**Results:** Intrathecal production of LCN2 was increased predominantly in patients with progressive MS ( $p < 0.005$  vs relapsing-remitting MS) and displayed a positive correlation to neurofilament light ( $p = 0.005$ ). Levels of LCN2 in brain microdialysates were severalfold higher than in the CSF, suggesting local production in progressive MS. Treatment with natalizumab in progressive MS reduced LCN2 levels an average of 13% ( $p < 0.0001$ ). LCN2 was found to inhibit remyelination in a dose-dependent manner in vitro.

**Conclusions:** LCN2 production is predominantly increased in progressive MS. Although this moderate increase does not support the use of LCN2 as a biomarker, the correlation to neurofilament light and the inhibitory effect on remyelination suggest that LCN2 might contribute to neurodegeneration through myelination-dependent pathways. *Neurol Neuroimmunol Neuroinflamm* 2016;3:e191; doi: 10.1212/NXI.0000000000000191

## GLOSSARY

**BSA** = bovine serum albumin; **DIV** = days in vitro; **EAE** = experimental autoimmune encephalomyelitis; **INDC** = inflammatory neurologic disease controls; **LCN2** = lipocalin-2; **MD** = microdialysates; **MOG** = myelin oligodendrocyte glycoprotein; **MS** = multiple sclerosis; **NFL** = neurofilament light; **PBS** = phosphate-buffered saline; **PPMS** = primary progressive MS; **RR** = relative recovery; **RRMS** = relapsing-remitting MS; **SC** = symptomatic controls; **SMI-31** = phosphorylated neurofilament; **SPMS** = secondary progressive MS; **TNF** = tumor necrosis factor.

The recent progress in the understanding of the pathophysiology and therapeutic options in multiple sclerosis (MS) pertains mainly to earlier relapsing-remitting MS (RRMS) stages. Our understanding of later disease stages is much more limited, and there is an urgent need to identify biomarkers of pathophysiologic pathways that can increase our knowledge and possibly lead to the identification of new therapeutic targets.<sup>1,2</sup>

Lipocalin-2 (LCN2) is a 25-kDa protein that was first identified as an acute phase protein stored and secreted by neutrophils.<sup>3,4</sup> It has now been ascribed multiple signaling roles, such as iron delivery, cell survival/death, differentiation, and inflammation, in physiologic and

Supplemental data  
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pathologic conditions.<sup>5</sup> Recently, a number of studies have pointed to a role for LCN2 in the CNS as well; in experimental models and cell culture systems, LCN2 induces reactive astrogliosis, neuronal migration, and death, and it also possibly has a detrimental effect on oligodendrocytes.<sup>6–10</sup> It also promotes M1 polarization of microglia and mediates their debranching and apoptosis.<sup>11,12</sup> Studies in experimental autoimmune encephalomyelitis (EAE) have suggested functional roles for LCN2, with regulatory effects on disease severity, proliferation of T cells, and demyelination.<sup>13–16</sup> Of note, LCN2 has been shown to be increased in a small cohort of patients with progressive disease (compared with RRMS).<sup>15</sup>

Therefore, in a case-control study, we compared the intrathecal production of LCN2 between patients with RRMS, patients with progressive MS, and controls, and the results prompted us to further investigate its regulation and potential functional relevance in progressive MS.

**METHODS Study design and patient samples.** We determined LCN2 levels in 3 different studies. First, we compared LCN2 production between patients with MS and controls and between patients with RRMS and progressive MS in a case-control study. CSF and plasma samples were obtained from an in-house biobank (Karolinska University Hospital, Sweden) containing samples collected during routine neurologic workups from 2003 to 2012. Demographic data of the patients included in this study are presented in table e-1 at [Neurology.org/nn](http://Neurology.org/nn). A total of 197 patients were included, of which 147 were patients with MS fulfilling the McDonald criteria (RRMS = 75 [relapse = 19, remission = 56]; secondary progressive MS [SPMS] = 49; primary progressive MS [PPMS] = 23). SPMS was defined as an initial relapsing-remitting disease course followed by more than 12 months of continuous worsening ( $\geq 0.5$  Expanded Disability Status Scale point) not explained by relapses. At time of sampling, none of the patients had received immunomodulatory treatment. Control groups were composed of symptomatic controls (SC) (n = 39; sensory symptoms = 34, dizziness/vertigo = 3, tension headache = 2) and inflammatory neurologic disease controls (INDC) (n = 11; systemic lupus erythematosus = 4, herpes encephalitis = 2, sarcoidosis = 1, anti-NMDA receptor encephalitis = 1, progressive multifocal encephalopathy = 1, demyelinating disease of unknown etiology = 1, myelopathy of unknown etiology = 1) according to the guidelines for biomarker studies in MS.<sup>17</sup> We used SC and INDC to investigate whether LCN2 can be used as a biomarker to distinguish between patients with MS and patients with similar neurologic symptoms and patients with similar CSF laboratory parameters, respectively. Second, we determined LCN2 levels in the CSF and microdialysates (MD) in a case series study of 7 patients with SPMS who received intrathecal delivery of rituximab (Umeå University, NCT01719159). In this study, microdialysis catheters were used to monitor the treatment effect. They were

inserted at baseline (day 0) in periventricular brain tissue and perfused with Plasmodes solution for 7 days. CSF was collected by lumbar puncture 1–2 days before the operative procedure and MD were collected 6 times a day. Finally, we measured the CSF levels of LCN2 in an open-label trial of natalizumab in progressive MS. In this cohort, LCN2 was determined in CSF collected from 10 patients with PPMS and 7 patients with SPMS at baseline and after 60 weeks of treatment with natalizumab. The details of the study design and outcome have been previously published.<sup>18</sup>

**Standard protocol approvals, registrations, and patient consents.** The regional ethical vetting boards of Stockholm (main study; 2003/2-548), Umeå (MD substudy; 2009/2107-31-2), and Copenhagen (natalizumab substudy; 2012-334-32M) approved the study procedures, and written informed consent was obtained from all patients.

**Measurements and relative recovery of LCN2 by microdialysis.** CSF samples were centrifuged (300g) immediately after sampling, aliquoted, and stored at  $-80^{\circ}\text{C}$  until analysis. Levels of neurofilament light (NFL) in the main CSF cohort were obtained from a previously published dataset.<sup>19</sup> LCN2 levels in CSF and plasma were measured using a commercially available ELISA kit (R&D Systems, Minneapolis, MN). Because LCN2 values reported in the literature were higher than those measured in our material,<sup>14,20</sup> we also ran approximately one-seventh of the CSF samples, including all groups of patients except for INDC, on an ELISA kit from Bioporto (Copenhagen, Denmark). The LCN2 levels measured with the Bioporto kit were 30% higher on average but correlated very well with the levels obtained from the R&D ELISA ( $R^2 = 0.93$ ) and were adjusted to the more conservative estimate. Measurements were optimized and performed for LCN2 using a 1:1.2 CSF to phosphate-buffered saline (PBS) dilution and a 1:200 plasma to PBS dilution. For the MD samples and their matched CSF obtained by lumbar puncture, the Bioporto ELISA was used because of the higher sensitivity and the need to work with higher dilutions because of the smaller collected volumes.

LCN2 is a molecule that exists in high concentrations in the blood, and passive leakage to the CNS may contribute to the levels detected in the intrathecal compartment. Therefore, we calculated the LCN2 index, which likely better reflects the intrathecal production of LCN2, according to the formula used to calculate the IgG index (i.e., LCN2 index:  $[\text{CSF LCN2}/\text{CSF albumin}]/[\text{plasma LCN2}/\text{plasma albumin}]$ ) and using samples obtained at the same time for each patient. CSF was available for LCN2 measurements from all patients. Patients for whom LCN2 index was not calculated because of random missingness of 1 or more of the other 3 parameters were excluded from the analyses (SC = 2, INDC = 2, RRMS = 3, SPMS = 18, PPMS = 5).

To estimate the real in vivo brain tissue concentrations of LCN2, the relative recovery (RR) of LCN2 by microdialysis was calculated in an in vitro experiment. Recombinant human LCN2 (Sigma-Aldrich, St. Louis, MO) was used for the RR experiment at concentrations 1 time, 10 times, and 100 times the maximum concentration obtained in the MD samples from patients. Recombinant LCN2 was diluted in Ringer solution with 0.2 mg/mL bovine serum albumin (BSA) to form an “artificial interstitial fluid” compartment. An MD catheter was immersed in the artificial solution and then perfused by Plasmodes solution by the same MD pump system as in the patients. After flush priming of the catheter, the MD fluid was drained to a waste tube for at least 40 minutes before sampling. Subsequently, MD were collected for 2 lots of 2 hours. The LCN2 concentrations in the

artificial solution and the collected MD were measured by ELISA (Bioporto).

**Myelinating cultures.** In vitro rat myelinating cultures were established as described previously.<sup>21</sup> Cultures were maintained at 37°C/7% CO<sub>2</sub> and fed thrice weekly by replacing half the culture medium with fresh differentiation media. After 12 days in vitro (DIV), insulin was omitted from the culture medium to promote myelination, with further culturing for up to 30 days. Myelinating cultures were either untreated or treated daily with 10 ng/mL, 100 ng/mL, or 1,000 ng/mL of recombinant rat LCN2 (R&D Systems) from 18 DIV (early) or 24 DIV (late) and for 10 to 6 days, respectively. Experiments were performed 3 times.

**Immunocytochemistry.** The following antibodies were used: mouse monoclonal SMI-31 (phosphorylated neurofilament, Abcam, Cambridge, UK), Z2 (anti-MOG [myelin oligodendrocyte glycoprotein]),<sup>22</sup> and rabbit polyclonal NG2 (chondroitin sulphate proteoglycan, Millipore, Billerica, MA). Secondary antibodies were labeled with Alexa Fluor 488 or Alexa Fluor 555 (Invitrogen, Waltham, MA). To visualize extracellular epitopes on live cells, primary antibody was applied for 30 minutes at 4°C. After repeated washing in ice-cold Dulbecco's modified eagle medium, subsequent steps were at room temperature. Cells were fixed in 4% paraformaldehyde for 20 minutes at room temperature. For cytoplasmic antigens, cells were permeabilized with 0.5% Triton X-100/PBS for 10 minutes (Sigma-Aldrich) followed by 1 hour in 1% BSA/10% normal goat serum/0.3M glycine. This was followed by application of primary antibodies for 1 hour, repeated PBS washing, application of appropriate secondary antibodies for 15 minutes, washing with PBS and distilled H<sub>2</sub>O, and mounting in Vectashield (Vector Laboratories, Burlingame, CA).

**Image analysis.** In each case, a minimum of 10 images (10× magnification) were acquired from 3 coverslips using an Olympus B×51 fluorescent microscope and Image-Pro software (Media Cybernetics, Rockville, MD). Axonal density was quantified using ImageJ software (NIH systems, version 1.41) as the relative area positive for SMI-31 (SMI-31<sup>+</sup>). To calculate the percentage of myelinated axons, MOG immunoreactive (MOG<sup>+</sup>) myelin sheaths were determined using the BRAINS BATCH algorithm, which uses pattern recognition software to distinguish between linear myelinated internodes and oligodendrocyte cell bodies. To quantify cell numbers, a minimum of 30 images were taken from 3 coverslips (20× magnification) and staining density was quantified using ImageJ. Cell counts were expressed as a percentage of the total NG2+ pixels within the total field.

**Statistical analyses.** Analyses were performed with Microsoft Excel and GraphPad Prism 5.0. Comparisons were done by 1-way analysis of variance with Bonferroni post hoc test. The mean values were used to calculate fold changes and are shown in the graphs. Correlation analyses were performed using the Pearson test.

**RESULTS Intrathecal production of LCN2 is increased in progressive MS and correlates to NFL.** Albumin quotient was significantly higher in patients with MS than in SC (1.25-fold), but there was no difference between MS subtypes. Plasma LCN2 was higher in INDC than in both SC and patients with MS (figure 1). The CSF LCN2 levels, as well as the LCN2 index, were higher (1.51- and 1.25- fold, respectively) in patients with MS than in SC (figure 2, A and B). Upon stratification for the MS

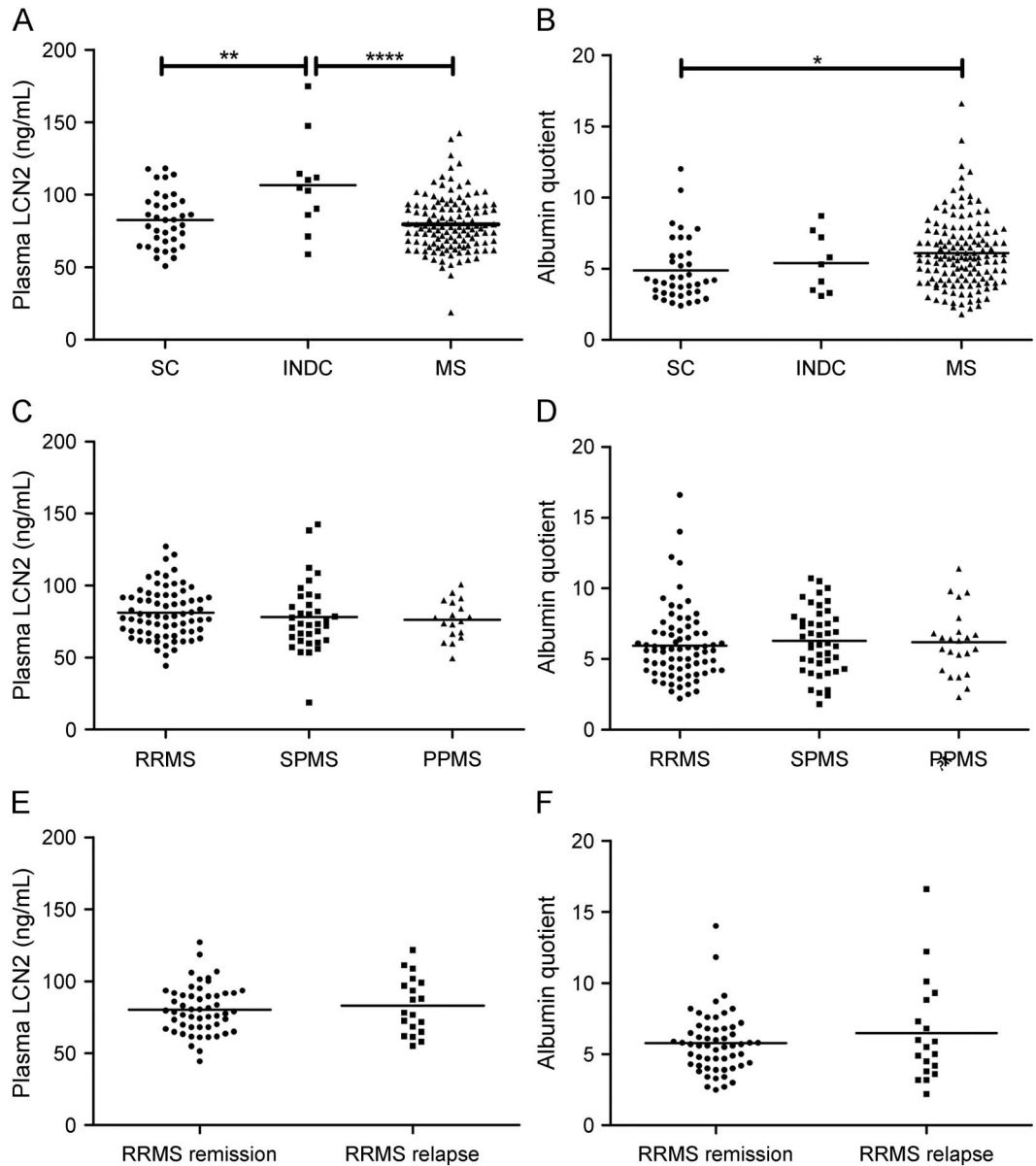
disease subtypes, we found that the CSF levels and the LCN2 index were higher in patients with SPMS (1.27- and 1.39-fold, respectively) and patients with PPMS (1.32- and 1.27-fold, respectively) than in patients with RRMS, although the LCN2 index comparison between PPMS and RRMS was not statistically significant (figure 2, C and D). The LCN2 index, but not CSF LCN2, was higher in remission than in relapse (figure 2, E and F). The higher levels of LCN2 in progressive MS were replicated in a separate cohort of 22 patients with RRMS and 24 patients with SPMS (1.39-fold,  $p < 0.001$ , data not shown). Because we found that LCN2 is increased in progressive MS, we next sought to study a possible functional role of LCN2 in human progressive disease and found that the LCN2 index correlated significantly to NFL (figure 3A). LCN2 did not correlate to age in all MS disease subtypes, and NFL levels were higher in RRMS than in SPMS (data not shown).

**LCN2 is found in high concentrations in in vivo brain tissue and its production is modestly reduced by natalizumab.** We next investigated whether the higher LCN2 levels reflect a local production in the CNS in progressive disease and whether LCN2 is regulated by adaptive immune pathways. Determination of LCN2 levels in MD collected at day 1, 2, and 3 after placement of MD catheters revealed much higher levels than those found in CSF from the same patients. RR as calculated by the in vitro experiment was 9% on average. This is similar to the RR measured in in vitro recovery studies with the 100-kDa cutoff MD membranes used for other molecules with similar molecular weights.<sup>23</sup> Collectively, the estimated local brain LCN2 concentrations were much higher than those in CSF, ranging from 13 times to 853 times the CSF values, which corresponds to concentrations in the range of 12–656 ng/mL (table 1).

Determination of LCN2 levels in patients with PPMS and SPMS participating in an open-label study with natalizumab<sup>18</sup> revealed a decrease of LCN2 60 weeks after treatment, but only by 13.9% on average (figure 3B).

**LCN2 inhibits myelination in neuroglial cell cocultures.** Because demyelination and impaired remyelination are important characteristics of MS pathophysiology, we next studied the effect of LCN2 in myelinating cultures. Addition of 100 ng/mL of LCN2 at 18 DIV inhibited myelination by 42.4%, whereas addition of 1,000 ng/mL resulted in a 78.9% inhibition (figure 4A). In contrast, exposure to 1,000 ng/mL of LCN2 at a later stage (24–30 DIV), when myelination in the cocultures had already occurred, did not affect myelination (figure 4B). LCN2 did not have

**Figure 1** Plasma LCN2 levels and albumin quotient in patients with MS and controls



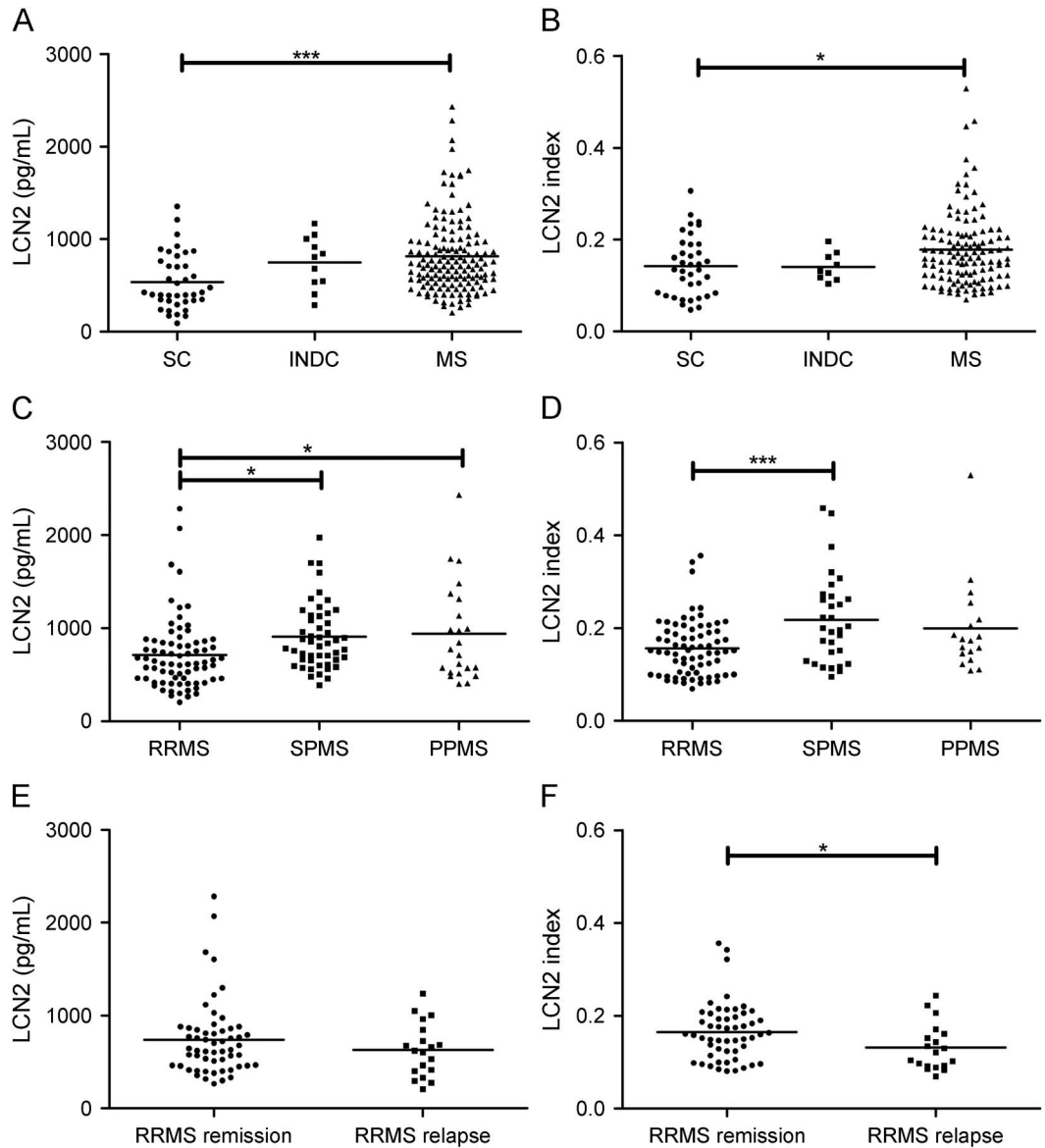
Plasma levels of lipocalin-2 (LCN2) and albumin quotient in (A, B) symptomatic controls (SC), inflammatory neurologic disease controls (INDC), and patients with multiple sclerosis (MS); in (C, D) patients with relapsing-remitting MS (RRMS), secondary progressive MS (SPMS), and primary progressive MS (PPMS); and in (E, F) patients in RRMS remission and RRMS relapse. \* $p < 0.05$ , \*\* $p < 0.01$ , and \*\*\*\* $p < 0.0001$ .

any effect on oligodendroglial progenitor cell numbers or axonal density (figure e-1).

**DISCUSSION** Because EAE studies suggested that LCN2 is a potentially important molecule for CNS autoimmunity, a biomarker for MS, and increased in the progressive form of MS, we measured LCN2 in a large case-control study of patients with MS and controls and found CSF LCN2 levels and intrathecal LCN2 production to be increased in patients with MS, predominantly those with progressive disease. This finding was subsequently replicated in an independent cohort of patients.

Our data are in accordance with the increased CSF LCN2 levels but not the increased plasma LCN2 levels reported previously in much smaller cohorts.<sup>14,15</sup> However, plasma LCN2 levels were increased in INDC, in line with previous publications.<sup>24,25</sup> However, the increase of the LCN2 index in patients with MS compared to controls and in progressive disease compared to RRMS is relatively moderate, with an overlap between the groups. This suggests that LCN2 is likely not a suitable biomarker for clinical diagnosis and prognosis; rather, its increase and correlation to NFL in SPMS indicate a functional role in this

**Figure 2** LCN2 protein levels in CSF and LCN2 index in patients with MS and controls



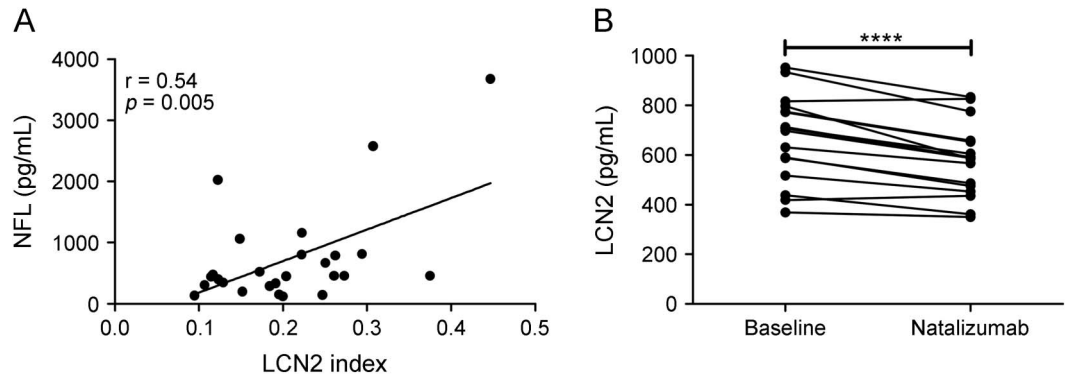
CSF levels of lipocalin-2 (LCN2) and LCN2 index values in (A, B) symptomatic controls (SC), inflammatory neurologic disease controls (INDC), and patients with multiple sclerosis (MS); in (C, D) patients with relapsing-remitting MS (RRMS), secondary progressive MS (SPMS), and primary progressive MS (PPMS); and in (E, F) patients in RRMS remission and RRMS relapse. \* $p < 0.05$  and \*\*\* $p < 0.001$ .

disease form. NFL is an axonal protein and a marker that is increased during all disease stages in MS; it is highest during relapses but is also increased in progressive disease and correlates with disease activity.<sup>26,27</sup> Therefore, the correlation between LCN2 and NFL is interesting because it is observed in a disease stage for which our knowledge about pathogenic processes is still limited. A detrimental effect of LCN2 on axonal degeneration in progressive MS can be induced by several mechanisms, as LCN2 has been shown to modulate CNS inflammation, induce neuronal cell death, regulate dendritic spine formation, and, importantly, modulate iron availability and transfer to the cells, which has been suggested to

be of particular importance for demyelination and neurodegeneration in progressive MS.<sup>6,13,20,28-31</sup>

We also describe an effect of LCN2 in inhibiting remyelination, but not inducing demyelination, in *in vitro* cell cultures in concentrations corresponding to *in vivo* brain tissue levels of 5 of 7 patients with progressive disease. This finding suggests that LCN2 might induce neurodegeneration through myelination-dependent pathways, a mechanism that has been well described in MS in previous studies.<sup>32,33</sup> Even though axonal density in cocultures was not affected by inhibition of remyelination through LCN2, it should be noted that it is difficult to translate acute treatment data in an experimental *in vitro*

**Figure 3** LCN2 correlation to NFL and natalizumab treatment effect on CSF LCN2 levels in SPMS



(A) Correlation between neurofilament light (NFL) and lipocalin-2 (LCN2) index in secondary progressive multiple sclerosis (SPMS). (B) Effect of 6 months of treatment with natalizumab on CSF LCN2 levels in progressive multiple sclerosis. \*\*\*\* $p < 0.0001$ .

model to chronic exposure over extensive time periods in the human brain tissue *in vivo*. The fact that LCN2 might inhibit remyelination is also important because impaired remyelination is a major component of MS pathology for which there are currently no good biomarkers or therapeutic options, although such treatments are in early stages of clinical trials.

In terms of the cause of the increased LCN2 levels in progressive MS, recent studies suggest a shift to a Th17-mediated response and also higher levels of tumor necrosis factor (TNF) in progressive MS, while both interleukin-17 and TNF may drive expression of LCN2 through effects on its promoter.<sup>34–37</sup> In an effort to further study the production of LCN2 and especially to dissect its regulation by adaptive vs innate immune pathways, we observed that treatment with natalizumab, which effectively targets lymphocyte migration into the CNS and drastically reduces NFL levels in RRMS,<sup>38</sup> had only a limited effect in terms of reducing LCN2 CSF levels. This finding suggests that production of LCN2 is in large part independent of adaptive immune responses and also highlights pathways of regulation other than those suggested by EAE studies, in which natalizumab drastically reduced LCN2 production in the CNS.<sup>14</sup> Neutrophils may also play a key role in this context, because neutrophils are a source of LCN2 and are

not blocked by natalizumab. In experimental models, neutrophils play a significant role in clinical onset of EAE, whereas their role in MS, although not clarified in detail, is suggested to be more prominent in later disease stages.<sup>39</sup> On the other hand, our data are in accordance with recent reports on experimental CNS disease models and human neuropathologic studies that show that infiltrating monocytes/macrophages and neutrophils, as well as astrocytes and neurons (but not lymphocytes), produce LCN2.<sup>6,14,15,20</sup> It will thus be important for future studies to investigate the immune cell or molecular pathways that regulate LCN2 in progressive disease to disclose additional pathophysiologic mechanisms that differ from RRMS.

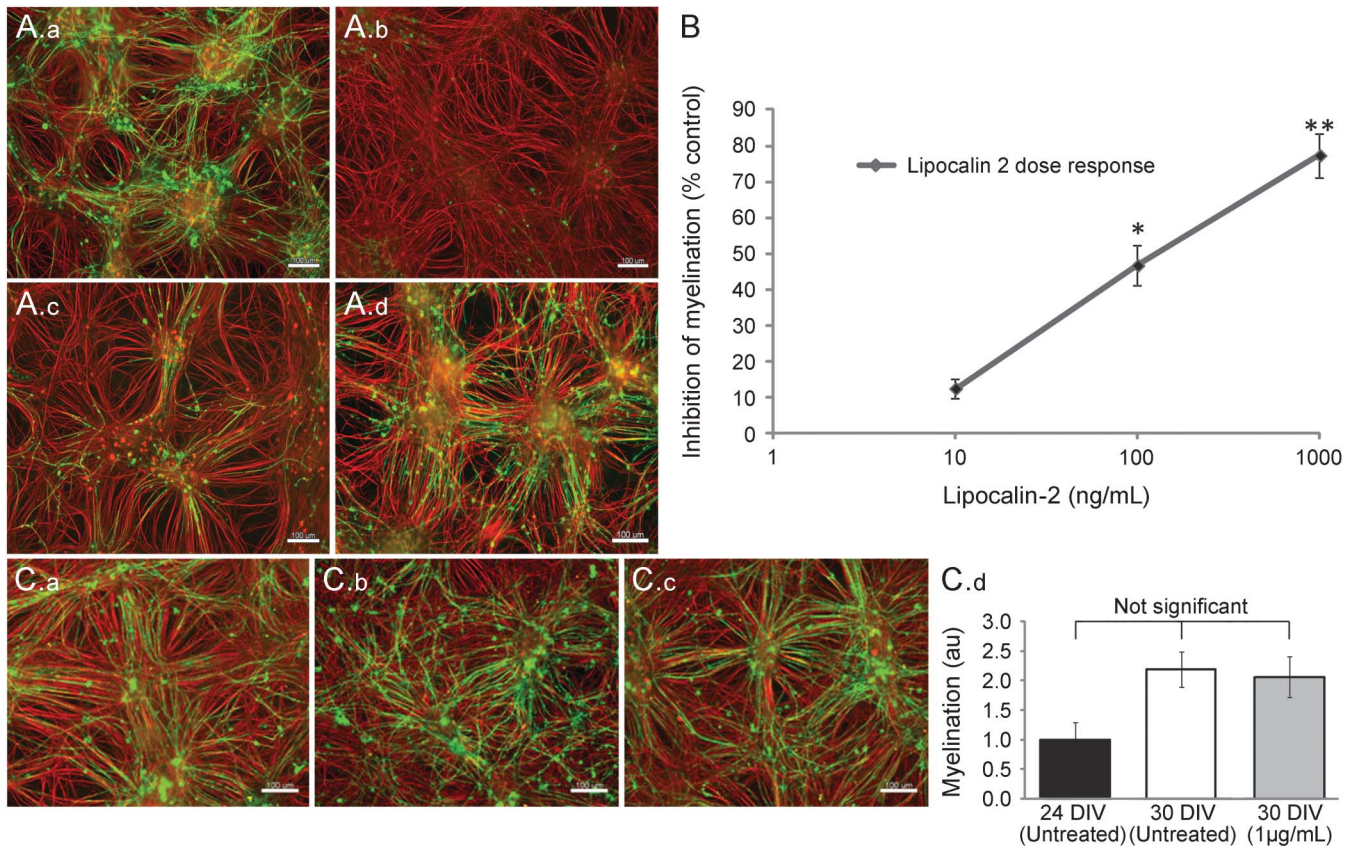
To further investigate the regulation of LCN2 *in vivo* in progressive MS, we measured LCN2 levels in MD and CSF and found them to be severalfold higher in brain interstitial fluid than in CSF in all 7 patients. In our MD measurements, we included 2 different time points from patient 2 (days 0 and 3) and patient 3 (days 1 and 3) and observed a temporal increase and decrease of LCN2 levels, respectively. This observed difference in kinetics possibly indicates that high local tissue production (not serum leakage and/or catheter-induced trauma) is the cause of high LCN2 levels. This is also supported by the fact that

**Table 1** LCN2 is severalfold higher in the *in vivo* brain tissue as seen by comparison of LCN2 levels in MD vs CSF

	Patient 1	Patient 2	Patient 2	Patient 3	Patient 3	Patient 4	Patient 5	Patient 6	Patient 7
Days after operation	1	0	3	1	3	3	2	1	1
MD LCN2	59,058	3,485	15,588	9,933	2,054	46,186	56,203	1,051	5,915
(MD LCN2)/RR	656,139	38,719	173,186	110,359	22,823	513,127	624,414	11,675	65,718
LP CSF LCN2	769	1,734		1,597		1,269	1,150	907	913

Abbreviations: LCN2 = lipocalin-2; LP CSF = CSF obtained via lumbar puncture; MD = microdialysates; RR = relative recovery.

**Figure 4** Early but not late exposure of myelinating cultures to LCN2 inhibits myelination



(A) Representative images taken from untreated myelinating cultures (A.a) or after treatment with 1,000, 100, or 10 ng/mL of lipocalin-2 (LCN2) (A.b–A.d, respectively) with quantification of immunochemical data (B). Untreated myelinating cultures at 24 days in vitro (DIV) (C.a), 30 DIV (C.b), or after addition of 1 µg/mL LCN2 at 24–30 DIV (C.c) with quantification of immunochemical data (C.d). phosphorylated neurofilament: red; myelin oligodendrocyte glycoprotein: green; scale bar = 100 µm. \**p* < 0.05, \*\**p* < 0.01.

the estimated MD LCN2 levels in some patients were severalfold higher than the serum values measured in the larger cohort. Because studies that have reported the levels of a molecule in both brain interstitial fluid and CSF in MS are rare, more research is needed to further explore the relationship between brain interstitial fluid and CSF in MS for LCN2 and other molecules and/or biomarkers. However, regarding LCN2, the severalfold higher levels in the interstitial fluid are not surprising in light of experimental data showing high local expression in the CNS and the described autocrine functions, and suggest that LCN2 is produced, secreted, and used in the brain tissue, with only a small fraction circulating in the CSF.<sup>7,11,15,40</sup>

We find that LCN2 is predominantly increased in the intrathecal compartment of patients with progressive MS and that this increase reflects a local tissue production, as indicated by the MD measurements in *in vivo* brain tissue. Furthermore, LCN2 inhibits remyelination *in vitro* and correlates to neurodegeneration in SPMS. These observations imply a detrimental role of LCN2 in progressive MS, where it is

locally produced in the CNS and might induce axonal degeneration, possibly through inhibition of remyelination. More studies in progressive disease models and/or MS to further elucidate the pathways that modulate the regulation and effect of LCN2 in progressive MS are warranted.

#### AUTHOR CONTRIBUTIONS

F. Al Nimer contributed to the design of the study, data collection, analysis and interpretation of the data, and drafting and writing the manuscript. C. Elliott contributed to the design of the study, data collection, analysis and interpretation of the data, and drafting and writing the manuscript. J. Bergman contributed to data collection and analysis and interpretation of the data. M. Khademi contributed to the design of the study, analysis and interpretation of the data, and revising the manuscript for intellectual content. A.M. Dring contributed to the design of the study, data collection, and analysis and interpretation of the data. S. Aceinehband contributed to data collection and analysis and interpretation of the data. T. Bergenheim contributed to the design of the study and analysis and interpretation of the data. J.R. Christensen contributed to the design of the study and analysis and interpretation of the data. F. Sellberg contributed to the design of the study and analysis and interpretation of the data. A. Svenningsson contributed to the design of the study, analysis and interpretation of the data, and drafting and revising the manuscript. C. Linington contributed to the design of the study, analysis and interpretation of the data, and drafting and revising the manuscript. T. Olsson contributed to the design of the study, analysis and interpretation

of the data, and revising the manuscript. F. Piehl contributed to the design of the study, analysis and interpretation of the data, and drafting and revising the manuscript.

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## DISCLOSURE

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