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ORIGINAL ARTICLE

Early central vs. peripheral immunological and neurobiological effects of fingolimod—a longitudinal study

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Abstract

Fingolimod (FTY) is known to have multiple effects on the immune system and the central nervous system (CNS) in patients with multiple sclerosis (MS). In this study, we evaluated the immunological and neurobiological effects of FTY in MS. Blood and cerebrospinal fluid (CSF) samples were collected from 15 MS patients before first FTY administration and after 4 months of FTY therapy. Immunophenotyping and evaluation of sphingosine-1-phosphate (S1P), neurofilament light chain (NFL), S-100 and neuron-specific enolase (NSE) levels were conducted. After 4 months of FTY therapy, absolute cell count in CSF was decreased from 6.33 to 2.43 MPt/l, accompanied by decreases of CD3+ (2.22 to 0.65 MPt/l) and of CD4+ counts (1.60 to 0.39 MPt/l). In blood, CD3+ (1.05 to 0.09 GPt/l), CD4+ (0.80 to 0.02 GPt/l), CD8+ (0.23 to 0.04 GPt/l) and CD19+ (0.21 to 0.01GPt/l) cell counts were as well reduced. CD14+ cell count remained stable over the same period (0.24 to 0.26GPt/l). NFL and S1P levels in CSF and blood were reduced over time (NFL: CSF 1759 to 1359 pg/l, blood 8.42 to 7.36 pg/l; S1P: CSF 2.12 to 0.71 nmol/l, blood 392.1 to 312.9 nmol/l). Strong correlations between CSF and blood NFL levels were observed. Neuronal damage markers such as S-100 (1.86 to 1.69 μg/l) and NSE (9.53 to 8.67 μg/l) were reduced to a lesser degree than other markers. FTY exerted significant effects on immunological and neurobiological markers in the central and peripheral compartment. Decreases in levels of neuroinflammatory and neurodegenerative markers were already evident after 4 months of treatment. Four-month serum NFL level appears to be a useful marker for FTY efficacy that correlates well with changes in the CNS compartment.

Key messages

- FTY has important immunological effects in both central and peripheral compartments.
- Cellular effects of FTY effects are more pronounced in the blood than in the CSF.
- FTY reduces S1P and NFL levels in CSF and serum.
- Serum NFL appears to be a useful marker for FTY therapy.

Keywords Multiple sclerosis · Fingolimod · CNS · S1P · CSF · Immune cell · Monitoring · Neurofilament light chain

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Background

Fingolimod (FTY) is approved for treatment for highly active relapsing-remitting multiple sclerosis (MS) [1]. FTY induces internalization of the sphingosine-1-phosphatase (S1P) receptor on lymphocytes [2, 3]. This results in an inhibition of lymphocyte egress from secondary lymphoid tissues along the existing S1P gradient into the blood [4, 5]. Immune cells that lack the homing C-C chemokine receptor type 7 (CCR7), which is important for recirculation into the lymph nodes, are not affected. As a result, the immunological changes in blood induced by FTY treatment are very specific [6, 7]. While these effects are well described for the peripheral blood compartment, there is only limited information regarding immunological effects inside the central nervous system (CNS) compartment of MS patients treated with FTY [8, 9].

In a range of clinical studies, FTY therapy has been associated with sustained effects in reducing brain volume loss, suggesting positive effects of FTY on neurobiological processes in MS patients [10]. Although the importance of non-imaging biomarkers has increasingly been recognised in recent years, no biomarkers of this type are routinely used to assess drug effects on neuroregeneration or neurodegeneration. Among potential candidate markers, neurofilament light chain (NFL) has been investigated for assessing axonal damage in MS patients using samples from cerebrospinal fluid (CSF) and blood [11, 12]. Pivotal studies have shown a decrease of NFL concentration in CSF and/or plasma by FTY treatment [13, 14]. However, to date, data on other neuronal damage markers, such as S-100 and neuron-specific enolase (NSE), are lacking.

Beyond effects on neurodegenerative markers, the effects of FTY on S1P metabolism are of particular interest because elevated S1P-levels have been identified in the CSF of patients with early-stage MS [15]. Although FTY interferes with the S1P receptor system, the effect of FTY on peripheral and central S1P levels in MS patients is not known. Moreover, chronic neuroinflammation (e.g. tick-born disease) has also been associated with alterations of S1P levels in CSF [16].

Several more direct and indirect effects of FTY are well known. The drug is discussed to have beneficial effects via reducing oxidate stress or reversing the downregulation of glutamate transporters in proinflammatory conditioned astrocytes [17, 18].

In this study, we conducted the first comprehensive, prospective analysis of immunological parameters in the central and peripheral compartment during the course of 4 months of FTY treatment in real-world clinical practice [19]. Additionally, neurobiological markers were investigated and evaluated for their utility as markers of treatment response. We selected the month 4 follow-up evaluation of CSF samples because of the real world setup at our MS centre.

Methods

Patients and study approval

The present study included 15 patients diagnosed with highly active MS at the MS centre Dresden, Germany (Table 1). CSF and blood were drawn before (baseline) and 4 months after the start of FTY therapy. Patients were required to be adults and fulfilling the label indication of FTY, defined as active disease despite full and adequate course of treatment with at least one disease-modifying therapy or two or more disabling relapses in 1 year, and with one or more gadolinium-enhancing lesions in brain MRI, or a significant increase in T2 lesion load as compared with previous recent MRI. All patients were required to provide written, informed consent. There were no exclusion criteria regarding disease duration or EDSS. The study was approved by the local ethical committee of the Dresden University of Technology (EK 348092014).

Sample processing and quantification of cells in CSF and blood

CSF was obtained by sterile lumbar puncture. Total CSF cell count was determined using a Sysmex XN5000 automatic analyser (Norderstedt, Germany). For immunological analysis, CSF samples were centrifuged within 20 min and analysed by fluorescence-activated cell sorting (FACS). Supernatants were collected for determination of S1P, S-100, NFL and NSE concentrations.

Phlebotomy was conducted immediately following the lumbar puncture procedure. The numbers of total leukocytes in blood, as well as the individual numbers of lymphocytes, granulocytes and monocytes, were measured on the Sysmex XN5000 machine. Peripheral blood mononuclear cells (PBMC) were isolated using Ficoll-Paque (Amersham Biosciences) and analysed via FACS. Plasma was collected for quantifying S1P and NFL concentrations.

Immune cell phenotyping

Ex vivo isolated peripheral blood mononuclear cells (PBMC) and CSF cells were transferred to 96-well FACS plate and washed twice with FACS buffer (1% fetal calf serum in phosphate-buffered saline). The cells were stained with fluorescence-labelled monoclonal antibodies for human cell surface markers for 20 min at 4 °C in the dark. The marker panel was anti-CD3 APC-Cy7, anti-CD4 Amcyan, anti-CD8 Pacific Blue, anti-CD14 APC, anti-CD19 PE-Cy7, anti-CD138 FITC (BD Bioscience) and anti-CD45 PerCP (Dako). Anti-mIgG1 FITC (BD Bioscience) was used as an additional corresponding isotope control. These single FACS panels were developed to gain as much immunological data as possible out of the limited CSF sample size. Samples were

Table 1 Patient characteristics

	Sex	Age	BMI	Years MS	Prev Tx	ARR prev year	Months relapse	Baseline EDSS
1	m	32	20.2	12	Natalizumab	1	2	3.0
2	f	41	21.7	13	Glatiramer acetate	3	3	3.0
3	f	46	20.0	1	Interferon beta 1b	1	6	2.0
4	f	46	26.9	6	Interferon beta 1a	1	8	2.0
5	f	40	30.5	10	Natalizumab	3	1	5.0
6	f	45	22.3	14	None	4	5	4.5
7	f	41	22.9	0	None	2	2	1.5
8	f	43	29.0	19	Natalizumab	3	1	2.5
9	f	27	20.3	10	Interferon beta 1a	0	38	2.5
10	f	28	18.4	10	Interferon beta 1a	1	9	6.0
11	m	26	20.5	3	Interferon beta 1a	2	4	3.5
12	f	41	32.3	3	Glatiramer acetate	1	2	1.5
13	f	34	25.7	1	Glatiramer acetate	0	12	3.0
14	m	43	27.2	9	Interferon beta 1a	1	15	2.5
15	f	44	22.8	2	None	2	2	4.0

Patient characteristics of the MS patients including sex (m, male; f, female), body mass index (BMI) in kg/m^2 , years since MS diagnosis was met (years MS), previous disease modifying treatment (prev Tx), annual relapse rate 12 months before baseline (ARR), months since last relapse (months relapse) and expanded disability status scale (EDSS) at baseline

analysed on LSR II analyser (BD Bioscience). Data analysis was conducted using FACSDiva software (BD Bioscience). In forward scatter versus side scatter dot plots, dead cells and debris were excluded. Immunocompetent cell subsets were defined according to the following surface markers: total T cells, CD3+; T helper cells, CD3 + CD4+; cytotoxic T cells, CD3 + CD8+; monocytes, CD14+; total B cells, CD19+; and plasmablasts, CD19 + CD138 + .

Determination of NFL, NSE and S-100 in CSF

NSE and S-100 were determined using a chemiluminescence immunoassay kit (Diasorin S.p.A., Italy) that was analysed using a Diasorin Liaison Analyser. NFL levels in CSF were evaluated using an ELISA kit (UmanDiagnostics AB, Sweden) that was analysed at 450 nm on a PhOMo photometer (Anthos Mikrosystems, Germany).

Determination of NFL in plasma

NFL in plasma was determined using the established Quanterix assay [20]. NFL levels were measured using the Simoa technique via a Human NF-Light Kit (Quartered, Lexington, MA). The assay was run on a Simoa HD-1 instrument (Quanterix) using a two-step Assay Neat 2.0 protocol [21]. To prepare for the assay, a 96-well assay plate was filled with calibrators, plasma samples and controls at room temperature (Quanterix). A capture antibody-coated paramagnetic beads, biotinylated detector antibody, streptavidin-beta-galactosidase, resorfurin beta-D-galactopyranoside and buffers were then loaded into the Simoa HD-1 Analyser. In the two-step Neat immunoassay, the target antibody beads were combined with sample and biotinylated detector antibody in the same incubation step. Calibrators and diluted plasma samples (dilution 1:4) were measured in duplicate, and sample dilution was calculated by the instrument. Calibrators ranged from 0 to 500 pg/ml. Predefined control measurements proved validity of the calibration curve and testing procedure.

Determination of S1P in blood and CSF

The concentration of S1P was evaluated according to the method previously described by Min et al. [22]. In short, acidified methanol and internal standard (30 pmol of C17-S1P, Avanti Polar Lipids, Inc., Alabaster, USA) were mixed with 250 μl of CSF or plasma. The samples were ultrasonicated for 60 s in ice-cold water. Lipids were extracted after addition of chloroform, 1 M sodium chlroid (NaCl) and 3 M sodium hydroxide (NaOH). The alkaline aqueous phase which contains S1P was transferred into a new tube. The residues of S1P in the chloroform phase were re-extracted twice with methanol/1 M NaCl (1:1, *v*/*v*). After re-extraction, all the aqueous fractions were mixed. The S1P quantity was assessed indirectly after dephosphorylation to SFO using alkaline phosphatase (bovine intestinal mucosa, Fluka, Milwaukee, WA, USA). To increase the extraction yield of released SFO, chloroform was gently placed in the bottom of the tubes. The chloroform fraction, which contains dephosphorylated sphingoid base, was further washed three times using alkaline water (pH adjusted to 10.0 with ammonium hydroxide) and

then evaporated under a stream of nitrogen. The dried residues of lipids were re-dissolved in ethanol, converted to their o-phtalaldehyde derivatives and finally determined with a high-performance liquid chromatography (HPLC) system (Prostar, Varian lnc. Walnut Creek, USA), which was equipped with a fluorescence detector and a C18 reversed-phase column (OmniSpher 5, 4.6150 mm, Varian Inc. Walnut Creek, USA). The isocratic eluent composed of acetonitrile/water (9:1, *v*/*v*) under a flow rate 1 ml/min was used. The column oven (Varian Inc. Walnut Creek, USA) was used to maintain a column temperature of 33 °C.

Statistical analysis

Data are expressed as mean and standard error of the mean. The Wilcoxon matched pairs test was used to investigate differences between baseline and month 4 data. Correlations were determined using the Spearman rank correlation. $p < 0.05$ was considered statistically significant.

Results

Patient characteristics

15 MS patients with highly active disease course and differing pre-treatment conditions were included in our study (Table 1). The median (range)/mean baseline characteristics were age 41 (26–46)/38.5 years, body mass index 22.8 (18.4–32.3)/24.05 kg/m^2, time since MS diagnosis was met 9 (0–19)/7.53 years, annual relapse rate 12 months before baseline 1 (0–4)/1.67, months since last relapse 4 (1–38)/7.33 and EDSS 3.0 (1.5–6.0)/3.10. No patient demonstrated any clinical or MRI activity in the first year of FTY treatment (no evidence of disease activity [NEDA]-positive). Six patients having common cold and one patient having urinary infection were reported as adverse events. No patient developed a severe lymphopenia below 0.2 GPt/l.

Immunological effects in blood versus CSF within first 4 months of FTY treatment

Seven out of 15 patients demonstrated a pathologically increased CSF cell count ≥ 5MPt/l at baseline that normalised in four of the patients after 4 months of FTY treatment. Comparing the mean CSF cell count at baseline versus 4 months, we could detect a significant decrease from 6.33 to 2.43 MPt/l, $p = 0.009$. In parallel, in the peripheral compartment, blood leukocyte count decreased from 4.05 to 0.85 GPt/l, $p < 0.001$ (Fig. 1A). In the same time frame, lymphocyte counts decreased from 1.84 to 0.39 GPt/l, $p < 0.001$ (data not shown).

In particular, CD3+ counts decreased in CSF (from 2.22 to 0.65 MPt/l, $p = 0.02$) and blood (from 1.05 to 0.09 GPt/l, $p < 0.001$). While CD4+ counts decreased significantly in both compartments (CSF: from 1.60 to 0.39 MPt/l, $p = 0.01$; blood: from 0.80 to 0.02 GPt/l, $p < 0.001$), CD8+ cells showed a significant decrease in blood only (CSF: from 0.54 to 0.23 MPt/l, $p = 0.23$; blood: from 0.23 to 0.04 GPt/l, $p < 0.001$). Overall, the CD4/CD8 ratio was significantly changed in both compartments (CSF: from 4.10 to 1.38, $p = 0.002$; blood: from 3.67 to 0.63, $p < 0.001$). CD19+ cells were decreased in CSF but changes did not reach level of statistical significance (from 0.47 to 0.16 MPt/l, $p = 0.73$). In blood, the same cell type significantly reduced in quantity from 0.21 to 0.01 GPt/l, $p < 0.001$. No changes in CD138+ plasma blast titres were measurable either in CSF (from 0.019 to 0.012 MPt/l, $p = 0.23$) or in blood (from 0.029 to 0.027 GPt/l, $p = 0.89$). CD14+ monocyte levels were similarly stable in both compartments (CSF: from 1.14 to 0.52 MPt/l, $p = 0.09$; blood: from 0.24 to 0.26 GPt/l, $p = 0.47$) (see Fig. 1A).

The individual ratio between cell counts at baseline compared with month 4 FTY was calculated for all immune cell subsets to illustrate the various FTY-mediated changes (Fig. 1b). In CSF, the highest ratio was measurable for total cell count, CD3+ and CD4+ cells. For CD8+ and CD19+ cells in CSF, the calculated ratio was much lower. In comparison with CSF, the highest ratios in blood were present for leukocytes, CD3+, CD4+, CD8+ and CD19+ cells. For both compartments, no relevant changes in CD14+ cell count could be identified (see Fig. 1b).

There was no correlation between the degrees of cell count decrease in CSF in comparison with that in blood.

At baseline, 12/15 patients demonstrated type 2 oligoclonal bands, 2/15 patients demonstrated type 3 oligoclonal bands and in one patient type 1 oligoclonal bands were present. After 4 months, three patients switched oligoclonal bands status as follows: one patient from type 2 to type 3, one patient from type 3 to type 4 and one patient from type 1 to type 4.

Neurobiological markers within the first 4 months of FTY treatment

Over the 4-month treatment period, NFL levels were significantly decreased in CSF (from 1759 to 1359 pg/l, $p = 0.03$) and plasma (from 8.42 to 7.36 pg/l, $p = 0.009$) (see Fig. 2A a).

We identified a highly significant association between NFL concentrations in CSF and plasma at baseline ($r = 0.74$, $p = 0.002$) and after 4 months ($r = 0.81$, $p < 0.001$) of FTY therapy (data not shown). Moreover, the data demonstrated that the decrease of NFL levels between baseline and after 4 months in CSF and plasma was correlated between both compartments ($r = 0.58$, $p = 0.03$) (data not shown). Positive correlation was present between the ARR and CSF baseline levels of NFL ($r = 0.53$, $p = 0.04$), whereas correlation of ARR and blood

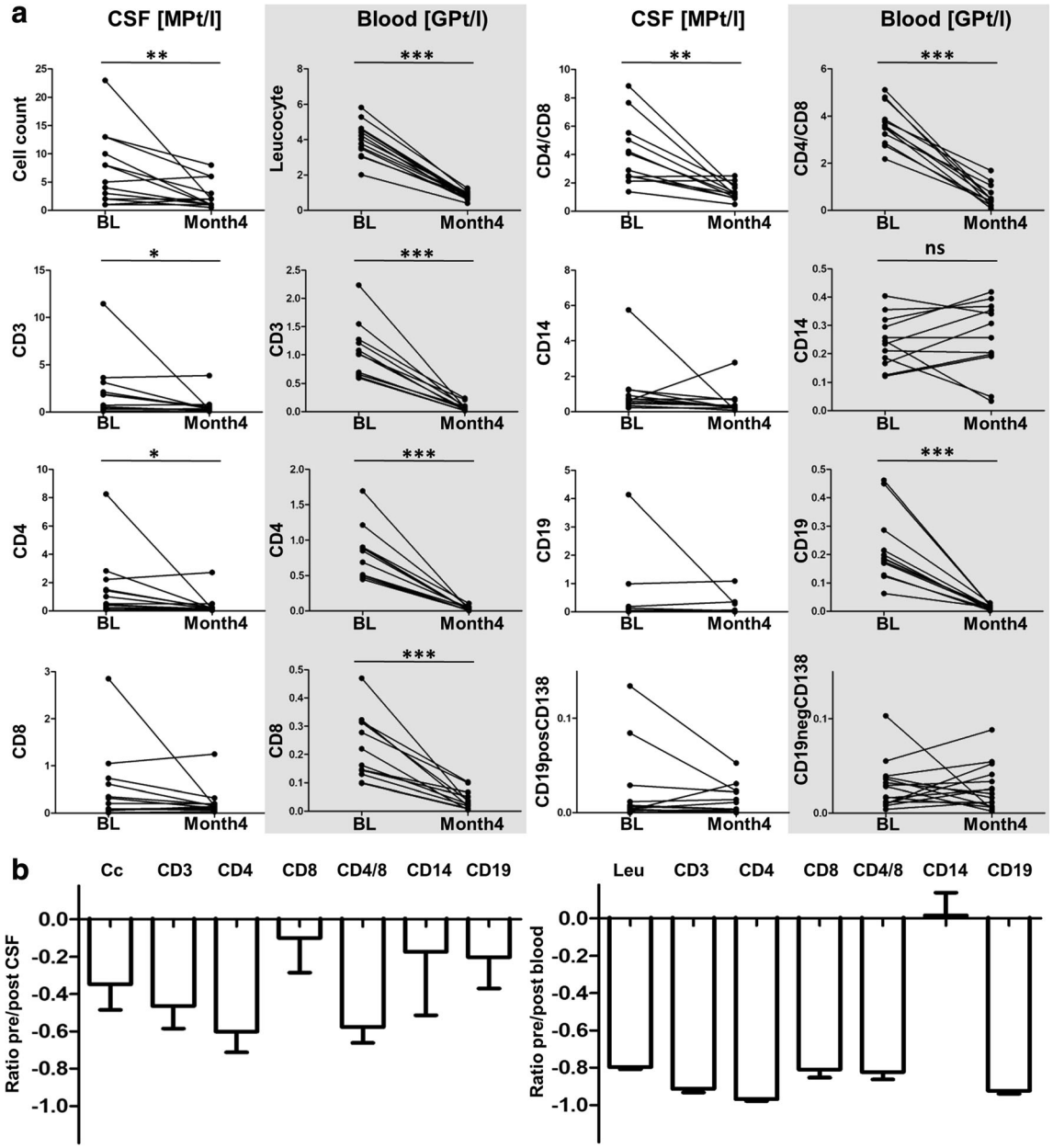

Fig. 1 Immunological markers. (**a**) Absolute cell counts of different immune populations and CD4/CD8 ratio in CSF (white) and blood (grey) compartments. Individual values at baseline and after 4 months of FTY treatment are presented by dots, and paired values are connected by lines. Asterisks indicate level of statistical significance (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). (**b**) For different immune subsets and CD4/CD8 ratio, the individual ratios of baseline and 4 months cell counts after start of FTY (pre/post) are displayed. Comparisons are represented as ratios of levels in CSF and blood compartments. Summarized data are shown as columns with standard errors. Cc, cell count

baseline levels of NFL narrowly failed to meet statistical significance ($r = 0.486$, $p = 0.07$) (data not shown). No correlations were present between baseline levels of CSF or blood NFL levels and the BMI ($r = 0.36$, $p = 0.19$; resp. $r = 0.13$, $p = 0.66$), years since MS diagnosis was met ($r = 0.09$, $p = 0.74$; resp. r = 0.15, $p = 0.59$), expanded disability status scale (EDSS) ($r = 0.14$, $p = 0.62$; resp. $r = 0.31$, $p = 0.26$) or months since last relapse ($r = -0.34$, $p = 0.21$; resp. $r = -0.46$, $p = 0.08$).

In selected patients, analysis of plasma NFL levels was conducted beyond 4 months of FTY treatment. Here, there was evidence of a sustained reduction of NFL levels (7.37 pg/l, $n = 8$ after 12 months, 5.66 pg/l, $n = 8$ after 24 months, data not shown).

In addition, there was a significant reduction of S1P levels in CSF after 4 months (from 2.12 to 0.71 nmol/l, $p = 0.004$). A smaller, but still significant reduction of S1P levels was evident in the peripheral compartment (from 392.1 to

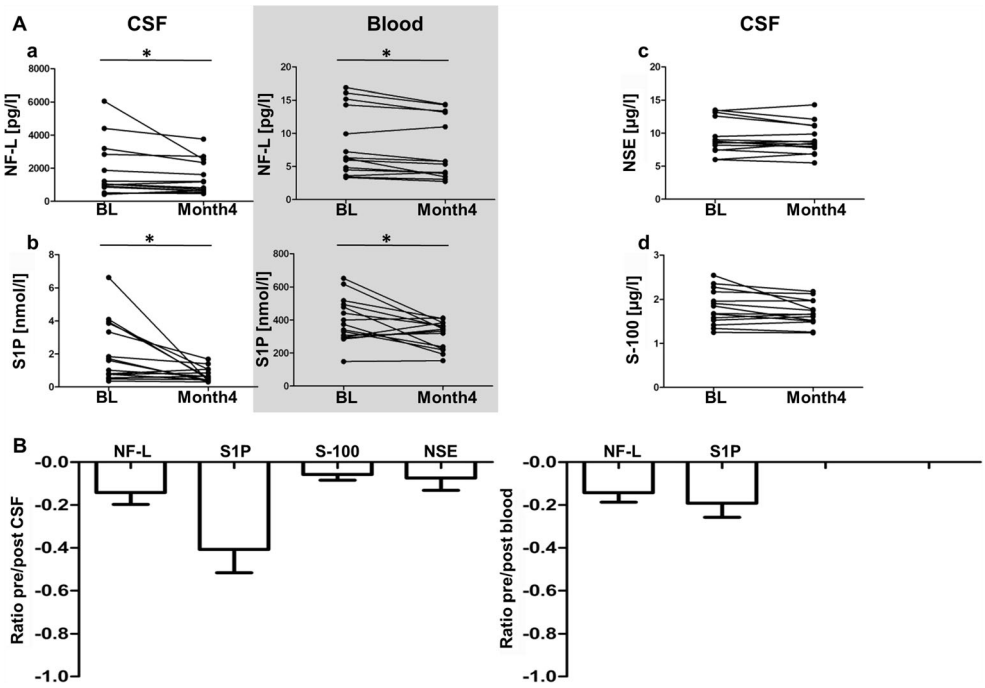

Fig. 2 Neurobiological markers. (A) Concentrations of different neurobiological markers in CSF and blood. Individual values at baseline and after 4 months of FTY treatment are presented by dots, and paired values are connected by lines. Asterisks indicate level of significance (*$p < 0.05$). (B) For different neurobiological markers the individual ratios of baseline and 4 months levels after start of FTY (pre/post) are displayed. Comparisons are represented as ratios of levels in CSF and blood compartments. Summarized data is depicted in bar columns and standard error

312.9 nmol/l, $p = 0.03$) (see Fig. 2A b). Other CSF markers of neuronal destruction, including NSE (from 9.53 to 8.67 µg/l, $p = 0.18$) and S-100 (from 1.86 to 1.69 µg/l, $p = 0.05$) showed a non-significant decreasing trend (see Fig. 2A c, d).

Correlations between CSF and blood levels of S1P were not evident at baseline or after 4 months of FTY treatment ($r = 0.31$, $p = 0.26$ resp. $r = -0.38$, $p = 0.16$). In the present study cohort, no correlations for ARR with S1P (CSF; $r = 0.13$, $p = 0.64$, plasma; $r = -0.27$, $p = 0.33$), S-100 ($r = -0.03$, $p = 0.91$) or NSE ($r = 0.08$, $p = 0.79$) were evident (data not shown). No correlations were present between baseline levels of CSF or blood S1P levels and the BMI ($r = 0.04$, $p = 0.88$; resp. $r = 0.43$, $p = 0.11$), years since MS diagnosis was met ($r = 0.58$, $p = 0.06$; resp. $r = -0.05$, $p = 0.87$), expanded disability status scale ($r = 0.41$, $p = 0.13$; resp. $r = 0.11$, $p = 0.70$) or months since last relapse ($r = 0.01$, $p = 0.96$; resp. $r = 0.46$, $p = 0.08$). As well, no correlations were present between levels of S-100 or NSE and the BMI ($r = -0.27$, $p = 0.33$; resp. $r = -0.35$, $p = 0.20$), years since MS diagnosis was met ($r = 0.19$, $p = 0.51$; resp. $r = 0.11$, $p = 0.69$), expanded disability status scale ($r = -0.22$, $p = 0.43$; resp. $r = -0.22$, $p = 0.42$) or months since last relapse ($r = -0.12$, $p = 0.67$; resp. $r = -0.28$, $p = 0.31$).

We calculated the individual ratios of NFL, S1P, NSE or S-100 between baseline and after 4 months. During therapy, the highest ratio—representing highest amount of change due to FTY treatment—was present for S1P levels in CSF, while the ratio in blood was less pronounced. The amount of decrease in NFL levels was comparable between both compartments. NSE and S-100 were measured only in CSF and showed low ratios (Fig. 2B).

Discussion

The present study has investigated clinical, immunological and neurobiological parameters in a well-characterized cohort of 15 MS patients. The aim was to evaluate the early effects of FTY treatment on neuroinflammatory and neurodegenerative markers. As per the European label for FTY, highly active relapsing-remitting MS patients with a mean ARR of 1.67 before FTY treatment were selected. All patients were characterised as responders as no clinical or MRI activity could be identified within the first year of FTY treatment.

FTY is functional antagonist of the S1P receptor, and thereby inhibits the egress of immune cells from lymphoid organs into the blood. This results in a range of immunological effects [1]. Our results—through with limited statistical power because of the small cohort size—give new information about early central treatment effects in FTY-treated MS patients. At present, only a few data are available on CSF immune cell distribution in FTY-treated MS patients. We could demonstrate a highly significant decrease of initially elevated total CSF cell count as well as significant decreases of CD+3 and CD4+ cells and the CD4/CD8 ratio. In a cross-sectional study, Kowarik et al. investigated relative cell frequencies in CSF [8], but these were not longitudinal data. A recently published study that examined longitudinal data from patients switching from natalizumab to FTY found a change in CD4/CD8 ratio comparable with the results of the present study [23]. For CD14+ and CD19+ cells, no studies have identified significant reductions in response to FTY therapy.

The longitudinal, parallel analysis of CSF and blood described here demonstrated that the chief effects on lymphocyte distribution occur in blood as opposed to CSF. Accordingly, CD3+, CD4+, CD8+ and CD19+ titres decreased over time, and this has been shown in previous studies [6, 7, 24]. Among B cells, the CD138+ plasmablasts were relatively resistant to FTY, which is in line with the results of Nakamura et al. [9]. These authors showed that CD138+ plasmablasts are enriched with chemokine receptor CXCR3, which would imply involvement of another migration pathway unaffected by FTY.

The present study did not demonstrate a significant direct link between blood and CSF changes, but it can be assumed that the CSF effects are mostly secondary to the pronounced changes in the immunological periphery that leads to normalization of cell counts, especially in patients with pathological CSF cell titres. Looking at the different immune subsets, CD4+ cells were more reduced than CD8+ cells in periphery, and a similar distribution was evident in the CSF. For CD14+ cells, stable cell counts were evident in both compartments. B cells showed a different effect: they were substantially reduced in number in the immunological periphery, but showed only a trend of decreasing numbers in the CSF. There may be other drug-mediated effects on central distribution in common with those implied for CD138+ plasmablasts [9]. It is possible that effects concerning blood-brain barrier could account for these differential effects [25].

Investigation of soluble serum and CSF markers could reveal potential markers of treatment response to FTY. In this pilot study, we included only NEDA-3 positive responders to FTY treatment, so no conclusion could be drawn regarding the differentiation of responders and non-responders using markers of this type.

NFL is a subunit of neurofilament and a neuronal cytoskeleton protein. Following axonal damage, NFL is released in the extracellular space [25]. In MS, NFL levels in CSF have been shown to correlate with MRI lesion load and disability scores [12]. Moreover, NFL serves as a marker of treatment response to therapies including FTY [26, 27]. With SIMOA technology, it was possible to detect NFL in blood. Recent studies have shown that CSF and serum/plasma levels of NFL are highly significantly correlated with each other, thereby presenting the possibility of blood NFL levels as marker of disease and treatment response in MS [12, 28, 33].

In the data described here, there was evidence of significant and correlated decreases in NFL levels in CSF and blood. NFL in CSF at baseline and previous ARR were also correlated, indicating the potential of NFL as neuroinflammatory marker.

The present study is the first time S1P has been investigated as a potential treatment marker in MS. While the origin of circulating S1P is uncertain and deserves further studies, it is known as a pleiotropic mediator with a role in inflammatory conditions. Latest since the approval of FTY in MS, a huge number of basic science studies of S1P and the S1P receptor axis have been conducted [27]. S1P is reported to have critical functions in angiogenesis, cardiovascular processes, neuronal development and immunity [29]. Kulakowska et al. showed elevated S1P levels in CSF in MS patients with early-stage disease in comparison with disease-free controls [15].

A greater degree of inflammation in the CNS may be responsible for elevated S1P CSF levels. Certainly, S1P-mediated signalling in CNS has been reported for astrocytes, neurons, oligodendrocytes and microglia—all of which are relevant to MS pathology [30], and FTY treatment may have an effect on any or all these cell types. In the study described here, a significant decrease in S1P levels in CSF and blood was evident after 4 months of FTY treatment. The decrease was more pronounced in the CSF compartment than in the blood. In contrast to NFL levels, no correlations between S1P CSF and blood levels were identified. S1P, with a direct role in the mechanism of action of FTY, could be a useful marker of FTY efficacy.

The established pleiotropic functions of S1P in health and disease make the interpretation of our results difficult. Another study has demonstrated elevated S1P levels in inflamed tissues as a migratory attractant [31], which would be reversed by FTY therapy. Additionally, a recent a study has shown a significant reduction of S1P receptor expression on different blood lymphocyte populations that would further decrease the attraction by tissue S1P [32]. Further studies are needed to evaluate whether S1P modulation in CSF or blood could serve a biomarker in MS disease in general, or only in MS patients treated with FTY.

While the focus of our observational study was set to the investigation of cellular and neurobiological effects, only a few correlations could be found with clinical or imaging outcomes. Repeated analysis over a longer time period after initiation of FTY would improve such analysis as has been done by us using real world lab data from clinical practice [34]. A further limitation of our study is the small study size which was caused by the invasiveness of the lumbar puncture procedure. Moreover, a control group of untreated MS patients or MS patients treated with other medication would help to emphasize that the demonstrated effects are FTY related. The present analysis only included FTY responders, and it would be important to repeat these assessments in non-responders.

Conclusion

In conclusion, significant immunological and neurobiological effects are evident after 4 months of FTY therapy in patients

with MS. NFL is proposed as a potential marker of FTY efficacy in these patients.

Compliance with ethical standards

Conflict of interests TS, RB and MZP have nothing to declare. KA received personal compensation from Novartis, Biogen Idec, Teva, Sanofi and Roche for the consulting service. TZ received personal compensation from Biogen Idec, Bayer, Novartis, Sanofi, Teva, and Synthon for the consulting services. Ziemssen received additional financial support for the research activities from Bayer, Biogen Idec, Novartis, Teva, and Sanofi Aventis.

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