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Permeability of the Blood–Brain Barrier Predicts No Evidence of Disease Activity at 2 Years after Natalizumab or Fingolimod Treatment in Relapsing–Remitting Multiple Sclerosis

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Objective: To investigate whether blood–brain barrier (BBB) permeability, as measured by dynamic contrast-enhanced magnetic resonance imaging (DCE-MRI), can provide early detection of suboptimal treatment response in relapsing–remitting multiple sclerosis (RRMS).

Methods: Thirty-five RRMS patients starting on fingolimod or natalizumab, drugs with a common effect of decreasing lymphocyte influx into the central nervous system, were scanned with DCE-MRI at 3T prior to treatment and at 3 and 6 months posttreatment. We calculated the influx constant Ki, a measure of BBB permeability, using the Patlak model. Suboptimal treatment response was defined as loss of no evidence of disease activity (NEDA) status after 2 years of treatment.

Results: Subjects with loss of NEDA status at 2 years had a 51% higher mean Ki in normal-appearing white matter (NAWM) measured after 6 months of treatment, compared to subjects with maintained NEDA status (mean difference = 0.06ml/100g/min, 95% confidence interval [CI] = 0.02–0.09, p = 0.002). Ki in NAWM at 6 months was a good predictor of loss of NEDA status at 2 years (area under the curve = 0.84, 95% CI = 0.70–0.99, p = 0.003), and a value above 0.136ml/100g/min yielded an odds ratio of 12.4 for suboptimal treatment response at 2 years, with a sensitivity of 73% and a specificity of 82%.

Interpretation: Our results suggest that BBB permeability as measured by DCE-MRI reliably predicts suboptimal treatment response and is a surrogate marker of the state of health of the BBB. We find a predictive threshold for disease activity, which is remarkably identical in clinically isolated syndrome as previously reported and established RRMS as investigated here.

A large number of immunomodulatory disease-modifying therapies (DMTs) are now available for relapsing–remitting multiple sclerosis (RRMS). Their main objective is a reduction in the number and severity of relapses, occurrence of new or enlarging lesions on magnetic resonance imaging (MRI), and prevention or delay in the onset of secondary progressive disease. In European countries, natalizumab and fingolimod share the same indication as second-line therapies in highly active RRMS, or as first-line therapy for aggressive and rapidly evolving disease.1 Natalizumab is a monoclonal antibody against the α4β1 integrin receptor, which mediates lymphocyte adherence to...
the endothelium, thereby directly suppressing lymphocyte passage across the blood–brain barrier (BBB). Fingolimod is an agonist of the sphingosine-1 receptor, inducing receptor internalization and thereby trapping encephalitogenic lymphocytes in lymph nodes, preventing them from migrating into the central nervous system. Hence, although the mechanism of action of these two drugs is different, their final effect is the same, that is, a reduction of the absolute number of lymphocytes trafficking across the BBB, as demonstrated by an equivalent reduction in CD4 lymphocyte counts in the cerebrospinal fluid (CSF). Both treatments have been shown to be highly efficacious in reducing relapse rates by 54 to 68%, reducing occurrence of new T2 lesions on MRI as well as the number of visibly contrast-enhancing lesions. Despite the high overall efficacy, the treatment response is highly heterogeneous, and a subset of patients still experience disease activity. To evaluate treatment response, the concept of no evidence of disease activity (NEDA), which uses a zero-tolerance threshold (no signs of disease activity in any of 3 domains) has been proposed as a treatment goal. Evaluating NEDA status after 2 years of DMT may be a reasonable approach, because it holds a positive predictive value of 78.3% for no progression at 7 years, with only minor improvement for re-evaluation at years 3 to 5. However, early detection of suboptimal treatment response is becoming increasingly important, both in the context of the increasing number of available therapies and due to DMTs seeming to have their best effect in the early stages of disease, but no current method or clinical variable exists that is able to perform such stratification.

We have previously reported that BBB permeability in multiple sclerosis (MS) normal-appearing white matter (NAWM), measured as the influx constant Ki by dynamic-contrast enhanced MRI (DCE-MRI), is abnormal when compared to controls, is a marker of recent clinical relapse activity, and is attenuated by disease-modifying treatment. Ki in NAWM correlates with biomarkers of immune cell trafficking in the CSF, and predicts conversion from optic neuritis to MS 2 years after onset. Hence, we hypothesize that Ki can stratify MS patients according to DMT response, here defined as loss of NEDA status at 1 and 2 years of second-line treatment. Furthermore, we aim to characterize the mechanistic relationship between Ki and cellular traffic, in the setting of treatments whose common end result is a reduction in lymphocyte traffic across the BBB.

**Patients and Methods**

**Study Participants**

We prospectively included all RRMS patients referred for MRI by the MS clinic at Rigshospitalet, Glostrup between August 2011 and November 2013 as part of an evaluation prior to initiation of natalizumab or fingolimod treatment. Inclusion criteria were: (1) an established diagnosis of MS, (2) clinical indication for treatment with either natalizumab or fingolimod, and (3) age = 18 to 59 years. Exclusion criteria were: (1) other concurring disease and (2) contraindication to MRI scan or MRI contrast agent. Eighty-five RRMS patients were assessed for eligibility, of whom 45 patients met the inclusion criteria and agreed to participate in a baseline scan (Fig 1). Thirty-five of these proceeded to initiation of either natalizumab or fingolimod, all of whom had a follow-up MRI performed at 3 months posttreatment. Twenty-nine patients participated in the 6-month posttreatment MRI. After study completion, 2 subjects were excluded, the first due to the occurrence of antinatalizumab antibodies, which resulted in treatment cessation after 5 months of treatment, and the second due to suspected side effects to fingolimod in the form of macular edema, which resulted in treatment cessation after 8 months. Follow-up MRI scans were performed as close as possible to the 3-month (mean = 97 days, standard deviation [SD] = 13 days) and 6-month time points (mean = 189 days, SD = 17 days) posttreatment using the therapy initiation day as reference and consisted of axial T2, axial fluid-attenuated inversion recovery (FLAIR), and axial postcontrast T1 of the cerebrum as well as DCE-MRI (see below for sequence parameters). Spinal cord assessment was performed at baseline, but not at the 3- and 6-month follow-up scans. We recorded any use of methylprednisolone during the course of the study, with the intention to postpone any 3- or 6-month follow-up scan by 2 months after completion of steroid treatment. Only 1 subject was treated with intravenous...
methylprednisolone during the first 6 months of the study. This occurred 4 months after treatment initiation, due to a major relapse 65 days before the planned 6-month scan, obviating the need for postponing the scan. Clinical data were obtained from hospital records 2 years after second-line treatment initiation for each individual subject. Collected variables were: baseline MS disease duration, baseline treatment status, history of methylprednisolone use, clinical relapses 12 months prior to second-line treatment initiation, and number of relapses, MRI activity, and Expanded Disability Status Scale (EDSS) at 1 and 2 years after treatment initiation. All subjects had regular clinical follow-up visits 3, 6, 12, 18, and 24 months post-treatment as part of standard clinical practice. Antimalarialumab antibodies were measured at 3, 6, 9, and 12 months. Collection of clinical data was performed by an experienced MS clinician who was blinded to the DCE-MRI results (another researcher analyzed the MRI data). Subjects who missed a clinical visit or an MRI were treated as missing data for the purpose of statistical analysis. Only subjects who completed a full 1 or 2 years of treatment were included in the 1- and 2-year analyses.

Outcome Measures
We used the following definitions. Relapse was defined as the appearance of new neurological symptoms or signs that lasted >24 hours in the absence of concurrent fever or illness. The treating physician recorded relapses at the face-to-face visits at 3, 6, 12, 18, and 24 months. Progression was defined as an EDSS score increase of 1 or more points recorded at a biannual visit.11 That was sustained at the subsequent clinical visit.11,19 If the EDSS score was zero at baseline, progression was defined as an EDSS score change of 1.5 or more that was sustained at the subsequent clinical visit.11 MRI activity was defined as new or enlarging T2 hyperintense lesions or T1 gadolinium-enhancing lesions in brain or spinal cord. To qualify as no evidence of MRI activity, new T2 hypointense lesions and T1 gadolinium-enhancing lesions had to be absent on brain and spinal cord MRI. As recently suggested, disease activity occurring within the first 3 months after initiation of natalizumab or fingolimod treatment was disregarded when assessing NEDA status, to allow for development of a full treatment effect.10,20 The earliest occurring loss of NEDA events within the 3 NEDA subdomains were: (1) a new T2 lesion at 6 months (this subject had another new T2 lesion at 1 year thus also fulfilling loss of NEDA at 12 months), (2) a relapse at 7 months, and (3) an EDSS increase at 1 year. Thus, loss of NEDA status did not occur prior to the 6-month MRI scan.

Ethics
This study was approved by the Ethics Committee of Copenhagen County according to the standards of the National Committee on Health Research Ethics, protocol number H-D-2008-002. All experiments were conducted in accordance with the Helsinki Declaration of 1975, and all subjects gave written informed consent.

DCE-MRI
MRI was performed on a 3T magnetic resonance unit (Achieva; Philips, Best, the Netherlands) using a 32-element phased-array head coil. DCE-MRI used a T1-weighted saturation-recovery gradient-echo sequence with flip angle = 30°, repetition time = 3.9 milliseconds, echo time = 1.9 milliseconds, centric phase ordering, parallel imaging factor = 2, acquired matrix = 96 × 61, acquired voxel size = 2.40 × 2.98 × 8mm³ (interpolated to 0.90 × 0.89 × 8mm³), field of view = 230 × 182mm², 5 slices, slice thickness = 8mm. Data for an initial measurement of relaxation time (T1) and equilibrium magnetization (M0) were generated using a series of saturation time delays from 120 milliseconds to 10 seconds, covering the same slices as imaged during the bolus passage. The dynamic sequence used a saturation time delay of 120 milliseconds, giving a time resolution of 1.25 seconds, and 750 time points, corresponding to a total sampling duration of 15.7 minutes. The automatic bolus injection (Spectris; MedRad, Warrendale, PA) with speed 3ml/s followed by 20ml saline was started after the 10th time point. The dose of contrast agent (gadobutrol 1mmol/ml) was 0.045mmol/kg body weight. We acquired a separate slice at the level of the internal carotid artery to obtain an arterial input function with minimal partial volume for every subject. The remaining 4 DCE slices were used for defining regions of interest (ROIs) and subsequent estimation of tissue pharmacokinetic values. To achieve a full clinical dose of gadobutrol (0.1ml/kg), which is important for adequate detection of visibly contrast-enhancing lesions,21 we injected the remaining contrast agent after the DCE acquisition and waited 5 minutes before acquiring the postcontrast T1 sequence.

MRI Sequences and ROIs
We used an axial T2-weighted MRI sequence (5 slices, echo time = 100 milliseconds, repetition time = 3,000 milliseconds, acquired voxel size = 0.57 × 0.76 × 8mm³ [interpolated to 0.45 × 0.45 × 8mm³], field of view = 230 × 119mm²) with same orientation and slice thickness (8mm) as our DCE-MRI sequence, to manually draw ROIs in the periventricular NAWM, and in the normal-appearing thalamic gray matter in both hemispheres, avoiding inclusion of, or proximity to, any MS lesions or diffusely abnormal white matter, as previously described in detail.10 Four ROIs were placed in periventricular NAWM (2 in the vicinity of the frontal ventricular horns [1 in each hemisphere] and 2 in the vicinity of the posterior horn [1 in each hemisphere]). Examples of ROI placement on anatomical images and corresponding Kᵢ maps from 2 subjects can be seen in Figure 2. T2 lesions counts were performed by an experienced neuroradiologist using an axial T2 FLAIR sequence (35 slices, echo time = 125 milliseconds, repetition time = 11,000 milliseconds, acquired voxel size = 0.65 × 0.99 × 3.5mm³ [interpolated to 0.45 × 0.45 × 3.5mm³], field of view = 230 × 119mm², slice thickness = 3.5mm). ROIs were placed a minimum of 10mm from any MS lesion or CSF-containing structures. In the presence of contrast-enhancing lesions on a postcontrast axial T1-weighted spin echo sequence (44 slices, echo time = 10 milliseconds, repetition time = 600 milliseconds, acquired voxel size = 0.94 × 1.25 × 3mm³ [interpolated to 0.94 × 0.94 × 3mm³], field of view = 240 × 240mm², slice thickness = 3mm), we took care not to
include the nearest 30mm of nonenhancing tissue. Our 4 DCE slices were placed with exactly the same angulation and anatomical position as the previous scan (evaluated for every scan). We ensured consistent positioning and size of our ROIs across different study time points by visual alignment with the previous scan.

### Permeability Estimation

The DCE-MRI data were analyzed with a semiautomated procedure using in-house MATLAB-based software. The DCE time series was converted to units of contrast agent concentration using T1 and M0, as determined from the multiple
satisfaction delay data, and a contrast agent relaxivity of $4s^{-1}/mM$. The input function was measured in the voxel of the internal carotid artery with maximal signal change during the bolus passage and was corrected for partial volume by normalizing to a magnitude- and phase-derived venous outflow function, sampled in the sagittal sinus ad modum Van Osch. The median signal–time curve for all voxels in the ROI was extracted and used to calculate permeability. For each tissue type, we used the median value of permeability to exclude effects of possible outliers (eg, 4 regions of NAWM were drawn, of which the median was used to represent NAWM). Every subject was represented by 1 value calculated as a mean of the tissue-specific ROIs, as previously described. 

**Statistics**

Histograms, probability plots, and modified Kolmogorov–Smirnov (Lilliefors) testing were used to analyze continuous variables for standard normal distribution fit. If the data were found to follow a normal distribution, 2-tailed Student t tests were used. If not, first a logarithmic transformation of the data was performed, and if normal distribution was not achieved, a Mann–Whitney U test was used. For comparisons between categorical data, chi-square tests were performed. We used a multiple linear regression approach to model the relationship between baseline Ki and MS clinical parameters. A 1-way repeated measures analysis of variance (ANOVA) was used to test for time effects after initiation of second-line treatment. Receiver operating characteristic (ROC) curves were used to estimate the predictive capability (area under the curve [AUC], and threshold with optimal sensitivity and specificity) of Ki to predict suboptimal treatment response, defined as loss of NEDA status. Logistic regression was performed to test for effects of multiple continuous independent variables on loss of NEDA status, and linear discriminant analysis was used when there were $>2$ possible outcomes. A $p$ value $<0.05$ allowed rejection of the null hypothesis. All analyses were performed in SPSS version 23 (IBM, Armonk, NY).

**MULTIPLE COMPARISONS.** The a priori hypothesis was that Ki after treatment initiation predicts suboptimal treatment effect, and we have thus investigated the performance of 4 different variables (Ki in NAWM and thalamus at 3 and 6 months). Applying a Bonferroni correction but taking the correlation coefficient (CC; average CC = 0.49) between the measured variables into account by way of the Dubey–Armitage–Parmar approach, the threshold for rejecting the null hypothesis becomes $p = 0.024$. All $p$ values are thus reported uncorrected, but only described as significant if falling below $p = 0.024$.

**Results**

**Baseline Data**

Univariate linear regression analysis showed that baseline permeability in NAWM was predicted by methylprednisolone treatment 2 months prior ($\beta = -0.50, p = 0.003$), but not by days since last relapse ($p = 0.38$), first-line treatment (yes/no; $p = 0.53$), or visibly contrast-enhancing lesions (whether entered as yes/no [$p = 0.70$] or actual count [$p = 0.68$]). In multivariate analysis, methylprednisolone treatment 2 months prior ($\beta = -0.71, p = 0.00008$) and days since last relapse ($\beta = -0.48, p = 0.005$) predicted baseline Ki in NAWM (model $R^2 = 0.40, p = 0.0002$), but not first-line treatment (yes/no; $p = 0.55$) or visibly contrast-enhancing lesions ($p = 0.76$). In thalamus, baseline Ki was predicted by methylprednisolone treatment 2 months prior to baseline ($\beta = -0.45, p = 0.01$), but not by days since last relapse ($p = 0.58$) in univariate analysis. Baseline permeability according to current treatment and recent relapse can be seen in Figure 3.
Treatment Effect

Between subjects receiving natalizumab and fingolimod there was no difference in mean Ki pretreatment (NAWM: mean difference = 0.008 ml/100g/min, 95% confidence interval [CI] = −0.05 to 0.06, p = 0.78; thalamus: mean difference = 0.01 ml/100g/min, 95% CI = −0.04 to 0.06; p = 0.65) and 6 months posttreatment (NAWM: mean difference = 0.004 ml/100g/min, 95% CI = −0.04 to 0.05, p = 0.84; thalamus: mean difference = 0.0002 ml/100g/min, 95% CI = −0.05 to 0.05, p = 0.99). However, at 3 months posttreatment, Ki in NAWM (mean difference = 0.06 ml/100g/min, 95% CI = 0.02–0.10, p = 0.002) and thalamus (mean difference = 0.04 ml/100g/min, 95% CI = 0.01–0.08, p = 0.011; both Student t tests) was higher in the natalizumab-treated patients, possibly reflecting a clinical selection bias favoring treatment of patients with highly active disease with natalizumab, as previously seen.32,33 Of natalizumab-treated subjects, 3 of 11 had a relapse during the first 6 months of treatment (occurring 7, 8, and 133 days posttreatment) as opposed to 1 of 24 fingolimod-treated subjects (occurring 20 days posttreatment), possibly reflecting the same bias. A 1-way repeated measures ANOVA analysis with Ki in NAWM pretreatment and 6 months posttreatment as outcome and baseline methylprednisolone, days since last relapse, and first-line treatment as covariates found no significant effect of time (p = 0.079), but the interaction between time and baseline methylprednisolone showed a trend (p = 0.041; Fig 4). Significant between-subject covariates were baseline methylprednisolone treatment (p = 0.001) and days since last relapse (p = 0.021).

No Evidence of Disease Activity

After 1 year of second-line treatment, 12 of 35 subjects (34%) lost NEDA status. After 2 years, this increased to 15 of 35 (43%). Five of 11 (45%) natalizumab-treated subjects and 10 of 24 (42%) fingolimod-treated subjects had lost NEDA status at 2 years. Of the 15 subjects who lost NEDA status at 2 years, 4 subjects had activity in all 3 NEDA subdomains (relapse[s], new MRI activity, and EDDS increase), 3 subjects had relapse(s) and EDDS increase, 1 subject had new MRI activity and EDDS increase, 4 subjects had relapse(s) only, 2 subjects had EDDS increase only, and 1 subject had MRI activity only. Baseline demographics, clinical characteristics, and Ki values according to NEDA status at 2 years are shown in Table 1. Three subjects experienced a relapse shortly after starting treatment (7, 8, and 20 days after treatment initiation), but per protocol these were disregarded. Subjects who lost NEDA status at 2 years had a 51% higher Ki in NAWM at 6 months posttreatment (mean difference = 0.06 ml/100g/min, 95% CI = 0.02–0.09, p = 0.002) and a 78% higher annual relapse rate (ARR) 1 year pretreatment (mean difference = 0.93, 95% CI = 0.38–1.5, p = 0.002; all Student t tests), when compared to subjects who maintained NEDA status (see Table 1 and Fig 5). Ki at baseline and 3 months in NAWM and thalami were nonsignificant between NEDA groups (NAWM baseline: mean difference = 0.013 ml/100g/min, 95% CI = −0.04 to 0.07, p = 0.62; thalamus baseline: mean difference = 0.02 ml/100g/min, 95% CI = −0.03 to 0.07, p = 0.39; NAWM at 3 months: mean difference = 0.016 ml/100g/min, 95% CI = −0.03 to 0.06, p = 0.45; thalamus at 3 months: mean difference = 0.02 ml/100g/min, 95% CI = −0.02 to 0.06, p = 0.25); thalamic Ki at 6 months showed an insignificant trend for higher values (mean difference = 0.043 ml/100g/min, 95% CI = 0.002–0.09, p = 0.040) in the loss of NEDA status group. In subjects who lost NEDA status at 1 year, only ARR 1 year pretreatment was significantly higher (mean difference = 0.99, 95% CI = 0.42–1.56, p = 0.001). An ROC curve with loss of NEDA at 2 years as outcome showed that Ki in NAWM at 6 months was a good predictor of loss of NEDA status at 2 years, with an AUC of 0.84 (95% CI = 0.70–0.99, p = 0.003; Fig 6). The optimal threshold, defined as the value that provided the highest...
| Characteristic                                      | NEDA Status at 2 Years | p   |
|---------------------------------------------------|------------------------|-----|
|                                                   | Lost, n = 15           |     |
|                                                   | Maintained, n = 20     |     |
| Age, yr                                          | 36 (8.2)               |     |
|                                                   | 43.1 (9.9)             | 0.03^a|
| Female gender, n                                 | 9 (60%)                |     |
|                                                   | 14 (70%)               | 0.72^b|
| EDSS score at baseline                           | 2.5 (1.6)              |     |
|                                                   | 3.2 (1.4)              | 0.17^a|
| Disease duration, yr                             | 4.7 (3.7)              |     |
|                                                   | 8.1 (6.8)              | 0.09^a|
| Number of relapses 1 year before treatment start | 2.1 (0.9)              |     |
|                                                   | 1.2 (0.7)              | 0.002^a,c|
| Last relapse onset, days                         | 150 (124)              |     |
|                                                   | 137 (110)              | 0.75^a|
| Relapse within 3 months from baseline            | 8 (53%)                |     |
|                                                   | 12 (60%)               | 0.74^b|
| Baseline treatment                               | 1.00^d                |     |
| None                                             | 3 (20%)                |     |
|                                                   | 5 (25%)                |     |
| Interferon-β                                     | 10 (67%)               |     |
|                                                   | 13 (65%)               |     |
| Glatiramer acetate                               | 2 (13%)                |     |
|                                                   | 2 (10%)                |     |
| Methylprednisolone < 2 months                    | 4 (27%)                |     |
|                                                   | 4 (20%)                | 0.70^b|
| Days since treatment end^e                        | 27 (23)                |     |
|                                                   | 39 (29)                | 0.80^a|
| Baseline MRI                                     |                         |     |
| T2 lesion count                                   | 19.1 (12.7)            |     |
|                                                   | 14.7 (8.5)             | 0.56^f|
| T2 lesion volume, mm^3                           | 14.5 (15.2)            |     |
|                                                   | 8.3 (3.5)              | 0.30^f|
| ≥1 Gd+ lesion                                    | 5 (33%)                |     |
|                                                   | 5 (25%)                | 1.00^b|
| Second-line treatment type = natalizumab         | 5 (33%)                |     |
|                                                   | 6 (30%)                | 1.00^b|
| K, NAWM, ml/100g/min                             |                         |     |
| Baseline, n = 35                                 | 0.148 (0.078)          |     |
|                                                   | 0.135 (0.072)          | 0.62^a|
| Size, voxels                                     | 163 (92)               |     |
|                                                   | 181 (102)              | 0.59^a|
| 3 months, n = 35                                 | 0.144 (0.049)          |     |
|                                                   | 0.129 (0.062)          | 0.45^a|
| Size, voxels                                     | 152 (92)               |     |
|                                                   | 161 (83)               | 0.76^a|
| 6 months, n = 28                                 | 0.166 (0.059)          |     |
|                                                   | 0.110 (0.029)          | 0.002^a,c|
| Size, voxels                                     | 170 (99)               |     |
|                                                   | 179 (86)               | 0.77^a|
| K, THAL, ml/100g/min                             |                         |     |
| Baseline, n = 35                                 | 0.152 (0.082)          |     |
|                                                   | 0.131 (0.057)          | 0.39^a|
| Size, voxels                                     | 129 (59)               |     |
|                                                   | 110 (51)               | 0.31^a|
| 3 months, n = 35                                 | 0.143 (0.042)          |     |
|                                                   | 0.125 (0.054)          | 0.25^a|
| Size, voxels                                     | 115 (43)               |     |
|                                                   | 130 (46)               | 0.33^a|
| 6 months, n = 28                                 | 0.165 (0.069)          |     |
|                                                   | 0.122 (0.037)          | 0.04^a|
| Size, voxels                                     | 143 (50)               |     |
|                                                   | 136 (47)               | 0.67^a|

Values are mean ± standard deviation. K, at 6 months and number of relapses before treatment start were significantly higher in subjects with loss of NEDA status at 2 years. Ki in thalamus at 6 months showed a trend for higher values but was nonsignificant.

^aStudent t test.
^bChi-square.
^cStatistically significant.
^dChi-square with first-line treatment yes/no.
^eOnly entered for subjects who received steroid treatment within the past 2 months.
^fStudent t test on log-transformed data.

EDSS = Expanded Disability Status Scale; Gd+ = Gadolinium enhancing lesion(s); MRI = magnetic resonance imaging; NAWM = normal-appearing white matter; NEDA = no evidence of disease activity; THAL = thalamus.
added sensitivity and specificity of Ki in NAWM for detecting loss of NEDA, was 0.136 ml/100g/min, providing a sensitivity of 73% and specificity of 82%. More than 1 annual relapse 1 year pretreatment predicted loss of NEDA (AUC = 0.79, 95% CI = 0.64–0.94, p = 0.004) with a sensitivity of 87% and specificity of 65%. Univariate logistic regression analysis showed that Ki in NAWM at 6 months was associated with loss of NEDA at 2 years (an increase of 1 SD [0.05 ml/100g/min] with an odds ratio [OR] = 10.4, 95% CI = 1.4–74, p = 0.02), as was number of annual relapses 1 year pretreatment (OR = 9.2, 95% CI = 1.8–48, p = 0.009), but not presence of active T2 lesions at 6 months, Ki in NAWM at 3 months, Ki in thalamus at 3 or 6 months, age, gender, MS years, EDSS, baseline lesion count, or baseline contrast-enhancing lesions. Multivariate analysis with all the above-mentioned covariates showed that Ki in NAWM > 0.136 ml/100g/min yielded an OR of 12.4 for loss of NEDA at 2 years, whereas > 1 annual relapse 1 year pretreatment was insignificant (Table 2). Two subjects switched to other therapies after 5 and 8 months, possibly influencing the NEDA outcome at 2 years. These were included in the primary analysis if they were on treatment while Ki was measured. Excluding these 2 subjects from the ROC curve analysis of Ki in NAWM at 6 months, with NEDA at 2 years as outcome, only caused minor changes to the results (AUC = 0.84, 95% CI = 0.70–0.99, p = 0.003, sensitivity 73%, specificity 81%).

**DCE-MRI versus Conventional Contrast Imaging**

Ten of 35 subjects (~29%) had 1 or more contrast-enhancing lesions on baseline MRI, and although these...
subjects had higher mean values of $K_i$ at baseline (NAWM, 0.15 ml/100 g/min; thalami, 0.15 ml/100 g/min), the difference was not significant when compared to subjects without contrast-enhancing lesions (NAWM, 0.14 ml/100 g/min; thalami, 0.14 ml/100 g/min; mean difference: NAWM, 0.01 ml/100 g/min, 95% CI = −0.05 to 0.07; thalamus, 0.01 ml/100 g/min, 95% CI = −0.03 to 0.05).

Only 2 subjects had contrast-enhancing lesions on the 3-month follow-up MRI, and no subjects showed contrast enhancement at the 6-month follow-up. We found no correlation between gadolinium-enhancing lesions at baseline and permeability at baseline, 3 months, or 6 months.

**Discussion**

**Mechanistic Insights**

This study enables investigation of the mechanistic relationship between $K_i$ (as measured by DCE-MRI) and cellular trafficking, by manipulation with disease-modifying treatments, which decrease cellular traffic across the BBB. We have previously found a correlation between $K_i$ and cellular traffic into the CSF, and absence of correlation between $K_i$ and albumin quotient, perhaps suggesting that $K_i$ may be a surrogate marker of cellular influx.17 This study addresses this issue, because the subjects maintaining NEDA represent an experimental situation where cellular traffic has been inhibited pharmacologically. We observe an apparent delay in the effect on $K_i$ such that 6-month but not 3-month $K_i$ predicted NEDA after 2 years. This lag is suggestive of an indirect process as opposed to an immediate effect of decreased cellular influx on $K_i$—which could reflect healing of the BBB solute barrier in the first 3 months after initiation of treatment. In those losing NEDA, one can hypothesize ongoing damage to the BBB. Hence, the association between cellular traffic and $K_i$ may not be direct, but more likely represents a sequence of events where changes in cellular influx predate changes in the physical integrity of the BBB solute barrier. Hence, solute BBB permeability appears to be a prognostic marker, by reflecting the “state of health” of the BBB. This study illustrates, in vivo, the fundamental difference between cellular and solute traffic in man. Dissociation between cellular traffic and solute permeability has been observed in vitro, where interferon beta reduces lymphocyte transmigration, while having no effect on the permeability to albumin.35

We have previously reported that a $K_i > 0.13$ ml/100 g/min identifies optic neuritis subjects with high risk of conversion to RRMS, adding significant value

| Variable                              | Optimal Cutoff$^a$ | Predicted, $n^b$ | Observed, n (% correct) | $p$  | Odds Ratio | 95% CI |
|--------------------------------------|--------------------|------------------|-------------------------|------|------------|--------|
| $K_i$ in NAWM 6 months posttreatment | $>0.136$ ml/100 g/min | 8 positives | 11 (73%) | 0.007 | 12.4 | 2 – 77 |
|                                      |                    | 14 negatives   | 17 (82%)                |      |            |        |
| $K_i$ in the thalamus 6 months       | $>0.124$ ml/100 g/min | 9 positives | 11 (82%) | 0.10 | —         | —      |
| posttreatment                        |                    | 11 negatives   | 17 (65%)                |      |            |        |
| Number of relapses 1 year            | $>1$               | 13 positives | 15 (87%) | 0.07 | —         | —      |
| before treatment start               |                    | 13 negatives   | 20 (65%)                |      |            |        |
| Baseline T2 lesion count             | $>13$              | 10 positives  | 15 (67%) | 0.94 | —         | —      |
|                                     |                    | 12 negatives  | 20 (60%)                |      |            |        |
| Active T2 lesions at 6 months        | $>0$               | 2 positives   | 11 (18%) | 0.06 | —         | —      |
|                                     |                    | 18 negatives  | 18 (100%)               |      |            |        |

Model Nagelkerke $R^2 = 0.37, p = 0.003$. $K_i$ in NAWM and thalamus are significant predictors of loss of NEDA status at 2 years. Number of relapses 1 year before treatment start showed a trend but was nonsignificant.

$^a$From receiver operating characteristic curve analysis.

$^b$Predicted loss of NEDA status.

CI = confidence interval; NAWM = normal-appearing white matter; NEDA = no evidence of disease activity.
compared to using T2 lesions alone. It is very interesting to note that the ROC threshold for further disease activity is identical in clinically isolated syndrome versus established RRMS. This solute BBB permeability threshold could act as a reproducible surrogate marker for a BBB state associated with active disease.

**Prediction of 2-Year NEDA Status**

We also report the novel finding that a single measurement of BBB permeability in NAWM performed 6 months after initiation of natalizumab or fingolimod is capable of predicting loss of NEDA status within the first 2 years of treatment. Because NEDA at 2 years has recently been shown to predict disability progression as measured by EDSS at 7 years nearly as well as NEDA at 5 years, measurements of BBB permeability could provide pivotal clinical information on treatment effect in the individual patient and possibly even provide long-term prognostic information. To our knowledge, no current method is capable of comparable stratification of treatment response to natalizumab or fingolimod. We find that $K_i$ in NAWM $> 0.136 ml/100g/min$ predicts loss of NEDA at 2 years ($OR = 12.4$). For comparison, $2$ contrast-enhancing lesions in the first year of treatment with interferon beta-1a identifies people at high risk of disability progression 15 years later. The OR for this effect was 8.9, one of the highest reported in the MS prediction literature.66

Presence of visibly contrast-enhancing lesions was not a significant determinant of $K_i$ in NAWM or thalamus at baseline, 3 months, or 6 months. Furthermore, $K_i$ in NAWM and thalamus was highly correlated in the same subjects at baseline (Spearman CC = 0.86), 3 months (CC = 0.88), and 6 months (CC = 0.82). Thus, the predictive effect of $K_i$ is unlikely to be a carryover from the prognostic effect of contrast-enhancing lesions or a result of spillover of contrast agent from enhancing lesions into the surrounding NAWM. Assuming a textbook value of water diffusion in brain tissue of $1.0 \times 10^{-9} m^2/s$,37 the distance a water molecule that has been in contact with contrast agent can diffuse during our DCE acquisition of 15 minutes is $10^{-9} m^2/15 \times 60 = 0.95 mm$. Thus, it is highly unlikely that water from a gadolinium-enhancing lesion would diffuse into our NAWM ROIs. However, one study found contrast-enhancing lesions in the first year of natalizumab treatment to predict future disease progression.38 In our cohort, contrast-enhancing lesions were only seen in 2 subjects at 3 months and none was observed at 6 months or at 1 year. Despite this, $K_i$ at 6 months predicted NEDA at 2 years. This highlights the value of DCE-MRI in the detection of diffuse low-level BBB leakage, as distinct from the focal high-level leakage detected by conventional contrast MRI, most likely reflecting the different pathological processes in MS lesions and NAWM. The acute MS lesion is characterized by demyelination, axonal damage, gliosis, lymphocyte and macrophage infiltrates, and focal BBB damage, whereas NAWM, despite retaining myelin, often exhibits axonal swelling, activated major histocompatibility complex II+ microglia and macrophages, gliosis, increased expression of proteolytic enzymes, and diffuse vessel leakage. DCE-MRI has the additional advantage of using a lower dose of gadolinium contrast compared to conventional contrast MRI.

Subjects with loss of NEDA at 2 years had significantly more relapses in the year preceding treatment initiation. However, baseline ARR and lesion count are not significant in the regression analysis of $K_i$ on NEDA; there is no correlation between 6-month $K_i$ and days since last relapse or baseline contrast-enhancing lesion count; we only observed 1 relapse in close proximity to the 6-month scan. This dispels the possibility that the predictive effect of the higher $K_i$ at 6 months on 2-year NEDA is a throwback to higher baseline disease activity.

**Possible Reasons for Treatment Failure**

We found that $K_i > 0.136 ml/100g/min$ predicts suboptimal natalizumab or fingolimod treatment response with a sensitivity of 73% and specificity of 81%. Possible reasons for treatment failure include: (1) lack of compliance, (2) neutralizing antibodies, (3) uncoupling of the disease process from the drug’s mechanism of action, and (4) high intrinsic disease activity. Lack of compliance and neutralizing antibodies were excluded in this study, because patients were monitored for both. Uncoupling of disease and drug mechanism of action may occur if the inflammatory process is self-driven within the brain or alternative pathways have developed that allow persistent encephalitogenic leucocyte entry into the brain, for instance, higher expression of human leucocyte antigen I, chemokines, and selectin ligands at the BBB and/or structurally damaged endothelium. When treatment failure is due to high intrinsic disease activity, the drug is effective at its target but the individual’s disease is so active that the therapeutic effect is not enough and disease activity breaks through.

$K_i$ Response

In our general linear model, we find a trend toward an interaction effect of time and baseline methylprednisolone treatment, indicating a treatment effect between paired baseline and 6-month $K_i$ only if baseline methylprednisolone is accounted for. This is indicative of a cumulative “$K_i$ response” to both types of treatment, that is, a decrease in $K_i$ at baseline due to methylprednisolone
and a decrease in $K_i$ during the course of treatment with fingolimod or natalizumab. Taken together, this indicates that $K_i$ response may provide a measure of treatment response. To further elucidate this relationship, one would need to follow individual patients over the course of several treatment regimes, encompassing both suboptimal and optimal treatment responses in the same individuals.

Propotion of NEDA

In this study, the proportion of subjects with loss of NEDA status was 34% during the first year and 43% during the second year. One natalizumab study reported loss of NEDA status at 2 years in 38% of subjects, but that study used less stringent MRI criteria not including enlarging T2 lesions. In the AFFIRM trial, 63% had lost NEDA status after 2 years of natalizumab treatment, using the same NEDA criteria as in our study. A head-to-head comparison of NEDA in natalizumab versus fingolimod treatment showed loss of NEDA status at 2 years in 30% and 77%, respectively. The large discrepancy in the proportion of subjects with loss of NEDA status could likely represent differences in patient selection. In clinical trials, subjects with highly active disease are often favored for inclusion, to show maximum effect of the treatment, whereas in this study we included all patients starting on natalizumab or fingolimod treatment during the given time window.

Permeability Changes in the Context of Natalizumab or Fingolimod Treatment

Soon et al investigated T1-weighted signal intensity changes after gadolinium–diethylenetriamine penta-acetic acid administration in 27 RRMS patients after 24 weeks on natalizumab treatment but found no effect of treatment on signal change in NAWM when compared to 13 patients receiving placebo. No clinical parameters, such as recent methylprednisolone treatment, relapses, and individual treatment effect, were taken into account; these are variables that we have shown govern $K_i$. This emphasizes the importance of including clinical covariates when characterizing changes in $K_i$ over time.

Solute permeability across the BBB, which is mainly governed by diffusion, is not synonymous with T-cell migration across the BBB, which is a highly regulated receptor-mediated process. To assess BBB permeability in this study, we used a macrocyclic gadolinium chelate (gadobutrol; Gd-BT-DO3A), which is a 547Da highly hydrophilic molecule. Thus, even though natalizumab blockage of the VLA-4 receptor results in reduced migration of T-cells across the BBB, this does not necessarily imply a change in solute permeability per se.

The time delay observed in this study of the effect of treatment on $K_i$ and the lack of correlation between baseline visibly contrast-enhancing lesions and $K_i$ at any time point, indicate that solute permeability in NAWM is secondarily modulated by a treatment-related reduction of low-grade inflammatory activity. In summary, we find that a single DCE-MRI at 6 months after initiation of natalizumab or fingolimod treatment provides information on the state of health of the BBB that enables reliable stratification of treatment response. Thus, DCE-MRI can enable early detection of long-term suboptimal treatment response in RRMS and a personalized medicine approach to treatment, a limitation being the long scan time (15 minutes). These results and the proposed thresholds require validation in larger studies.

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Author Contributions

Study concept and design: S.P.C., J.L.F., H.B.W.L. Data acquisition and analysis: S.P.C., H.J.S., J.L.F., H.B.W.L. Drafting the text and figures: all authors.

Potential Conflicts of Interest

S.P.C. has received research funding and travel funding from Biogen Idec. J.L.F. has served on scientific advisory boards for and received funding for travel related to these activities as well as speaker honoraria from Biogen Idec. H.B.W.L. has received research funding from Biogen Idec. Biogen Idec produces and benefit from sales of natalizumab, which was investigated in the present study. However, Biogen Idec had no influence on study setup, subject inclusion, data analysis, interpretation of results, or publishing decisions, and intellectual rights belong to the authors alone. H.J.S., I.G., and A.V. report no conflicts of interest for the present study.
References

1. Rio J, Comabella M, Montalban X. Multiple sclerosis: current treatment algorithms. Curr Opin Neurol 2011;24:230-237.
2. Yednock TA, Cannon C, Fritz LC, et al. Prevention of experimental autoimmune encephalomyelitis by antibodies against alpha 4 beta 1 integrin. Nature 1992;356:63-66.
3. Matloubian M, Lo CG, Cinamon G, et al. Lymphocyte egress from thymus and peripheral lymphoid organs is dependent on S1P receptor 1. Nature 2004;427:355-360.
4. Kowark MC, Pellkofer HL, Cepok S, et al. Differential effects of fingolimod (FTY720) on immune cells in the CSF and blood of patients with MS. Neurology 2011;76:1214-1221.
5. Polman CH, O’Connor PW, Havrdova E, et al. A randomized, placebo-controlled trial of natalizumab for relapsing multiple sclerosis. N Engl J Med 2006;354:899-910.
6. Kappos L, Heinrich MC, Rudick RA, et al. Treatment effective-ness of alemtuzumab compared with natalizumab, fingolimod, and interferon beta-1a for relapsing-remitting multiple sclerosis patients. Neurology 2011;76:1214-1221.
7. Cohen JA, Barkhof F, Comi G, et al. Oral fingolimod or intramus-cular interferon for relapsing multiple sclerosis. N Engl J Med 2010;362:402-415.
8. Rio J, Comabella M, Montalban X. Predicting responders to thera-pies for multiple sclerosis. Nat Rev Neurol 2009;5:553–560.
9. Lublin FD. Disease activity free status in MS. Mult Scler Relat Disord 2012;1:6–7.
10. Giovannoni G, Turner B, Gnanapavan S, et al. Is it time to target no evident disease activity (NEDA) in multiple sclerosis? Mult Scler Relat Disord 2015;4:329–333.
11. Rotstein DL, Healy BC, Malik MT, et al. Evaluation of no evidence of disease activity in a 7-year longitudinal multiple sclerosis cohort. JAMA Neurol 2015;72:152–158.
12. Kappos L, Havrdova E, Giovannoni G, et al. No evidence of dis-ease activity in patients receiving daclizumab versus intramuscular interferon beta-1a for relapsing-remitting multiple sclerosis in the DECIDE study. Mult Scler 2017;23:1736–1747.
13. Kalincik T, Brown JWL, Robertson N, et al. Treatment effective-ness of alemtuzumab compared with natalizumab, fingolimod, and interferon beta in relapsing-remitting multiple sclerosis: a cohort study. Lancet Neurol 2017;16:1222–1232.
14. Freedman MS, Abdoli M. Evaluating response to disease-modifying therapy in relapsing multiple sclerosis. Expert Rev Neurother 2015;5:407–423.
15. Rio J, Nos C, Tintoré M, et al. Defining the response to interferon-β1 in relapsing-remitting multiple sclerosis patients. Ann Neurol 2006;59:344–352.
16. Cramer SP, Simonsen H, Frederiksen JL, et al. Abnormal blood-brain barrier permeability in normal appearing white matter in multiple sclerosis investigated by MRI. Neuroimaging Clin N Am 2014;4:182–189.
17. Cramer SP, Modvig S, Simonsen HJ, et al. Permeability of the blood-brain barrier predicts conversion from optic neuritis to mul-tiple sclerosis. Brain 2015;138:2571–2583.
18. Schumacker GA, Beebe G, Kibler RF, et al. Problems of experi-mental trials of therapy in multiple sclerosis: report by the Panel on the Evaluation of Experimental Trials of Therapy in Multiple Sclerosis. Ann N Y Acad Sci 1965;122:552–568.
19. Havrdova E, Galetta S, Hutchinson M, et al. Effect of natalizumab on clini-cal and radiological disease activity in multiple sclerosis: a retrospective analysis of the Natalizumab Safety and Efficacy in Relapsing-Remitting Multiple Sclerosis (AFFIRM) study. Lancet Neurol 2009;8:254–260.
20. Stangel M, Penner IK, Kallmann BA, et al. Towards the implementa-tion of “no evidence of disease activity” in multiple sclerosis treatment: the multiple sclerosis decision model. Ther Adv Neurol Disord 2015;8:3–13.
21. Rovira À, Wattjes MP, Tintoré M, et al. Evidence-based guidelines: MAGNIMS consensus guidelines on the use of MRI in multiple sclerosis—clinical implementation in the diagnostic process. Nat Rev Neurol 2015;11:1–12.
22. Larsson HBW, Hansen AE, Berg HK, et al. Dynamic contrast-enhanced quantitative perfusion measurement of the brain using T1-weighted MRI at 3T. J Magn Reson Imaging 2008;27:754–762.
23. Hansen AE, Pedersen H, Rostrup E, Larsson HBW. Partial volume effect (PVE) on the arterial input function (AIF) in T1-weighted per-fusion imaging and limitations of the multiplicative rescaling approach. Magn Reson Med 2009;62:1055–1059.
24. Van Osch MJ, Vonken EJ, Bakker CJ, Viergever MA. Correcting partial volume artifacts of the arterial input function in quantitative cerebral perfusion MRI. Magn Reson Med 2001;45:477–485.
25. Cramer SP, Larsson HBW. Accurate determination of blood-brain barrier permeability using dynamic contrast-enhanced T1-weighted MRI: a simulation and in vivo study on healthy subjects and multiple sclerosis patients. J Cereb Blood Flow Metab 2014;34:1655–1665.
26. Larsson HBW, Courivaud F, Rostrup E, Hansen AE. Measurement of brain perfusion, blood volume, and blood-brain barrier perme-ability, using dynamic contrast-enhanced T1-weighted MRI at 3 tesla. Magn Reson Med 2009;62:1270–1281.
27. Barber TW, Brockway JA, Higgins LS. The density of tissues in and about the head. Acta Neurol Scand 1970;46:85–92.
28. Lilliefor WS. On the Kolmogorov-Smirnov test for normality with mean and variance unknown. J Am Stat Assoc 1967;62:399.
29. Dallal GE, Wilkinson L. An analytic approximation to the distribu-tion of Lillifors’s test statistic for normality. Am Stat 1986;40:294.
30. Sankoh AJ, Huque MF, Dubey SD. Some comments on frequently used multiple endpoint adjustment methods in clinical trials. Stat Med 1997;16:2529–2542.
31. Blakesley RE, Mazumdar S, Dew MA, et al. Comparisons of meth-ods for multiple hypothesis testing inneuropsychological research. Neuropsychology 2009;23:255–264.
32. Barbin L, Rousseau C, Doussel N, et al. Comparative efficacy of fingolimod vs natalizumab: a French multicenter observational study. Neurology 2016;86:771–778.
33. Kalincik T, Horakova D, Spelman T, et al. Switch to natalizumab versus fingolimod in active relapsing-remitting multiple sclerosis. Ann Neurol 2015;77:425–435.
34. Dou KH, O’Malley AJ, Mauri L. Receiver-operating characteristic analysis for evaluating diagnostic tests and predictive models. Circulation 2007;115:654–657.
35. Prat A, Biemacki K, Antel JP. Th1 and Th2 lymphocyte migration across the human BBB is specifically regulated by interferon-β and copolymer-1. J Autoimmun 2005;24:119–124.
36. Bemra R, You Y, Colica P, et al. Predictors of long-term out-come in multiple sclerosis patients treated with interferon beta. Ann Neurol 2013;73:95–103.
37. Le Bihan D, Lima M. Diffusion magnetic resonance imaging: what water tells us about biological tissues. PLoS Biol 2013;11:1–3.
38. Raffel J, Gafson AR, Dahdaleh S, et al. Inflammatory activity on natalizumab predicts short-term but not long-term disability in multiple sclerosis. PLoS One 2017;12:e0169546.
39. Ludwig SK. The pathogenesis of multiple sclerosis: relating human pathology to experimental studies. J Neuropathol Exp Neurol 2006;65:305–318.
40. Moll NM, Rietsch AM, Thomas S, et al. Multiple sclerosis normal-appearing white matter: pathology-imaging correlations. Ann Neurol 2011;70:764–773.
41. Plumb J, McQuaid S, Mirakhor M, Kirk J. Abnormal endothelial tight junctions in active lesions and normal-appearing white mat-ter in multiple sclerosis. Brain Pathol 2002;12:154–169.
42. Prosperini L, Fanelli F, Pozzilli C. Long-term assessment of no evidence of disease activity with natalizumab in relapsing multiple sclerosis. J Neurol Sci 2016;364:145–147.

43. Baroncini D, Ghezzi A, Annovazzi PO, et al. Natalizumab versus fingolimod in patients with relapsing-remitting multiple sclerosis non-responding to first-line injectable therapies. Mult Scler 2016;22:1315–1326.

44. Soon D, Altmann DR, Fernando KTM, et al. A study of subtle blood brain barrier disruption in a placebo-controlled trial of natalizumab in relapsing remitting multiple sclerosis. J Neurol 2007;254:306–314.

45. Bechmann I, Galea I, Perry VH. What is the blood-brain barrier (not)? Trends Immunol 2007;28:5–11.

46. Varatharaj A, Galea I. The blood-brain barrier in systemic inflammation. Brain Behav Immun 2017;60:1–12.

47. Saremi F. Perfusion imaging in clinical practice: a multimodality approach to tissue perfusion analysis. Alphen aan den Rijn, the Netherlands: Wolters Kluwer Health, 2015.

48. Engelhardt B, Coisne C. Fluids and barriers of the CNS establish immune privilege by confining immune surveillance to a two-walled castle moat surrounding the CNS castle. Fluids Barriers CNS 2011;8:4.