Research Article

Title: Permeability of the blood-brain barrier predicts no evidence of disease activity at two years after natalizumab or fingolimod treatment in relapsing-remitting multiple sclerosis

Running head: BBB permeability predicts treatment response in MS

[1]Stig P. Cramer, MD, PhD, [1]Helle J. Simonsen, [2]Aravinthan Varatharaj, BMBCh, [2]Ian Galea, MD, PhD, [3,4]Jette L. Frederiksen, MD, DMSc, [1,4]Henrik B.W. Larsson, MD, DMSc

[1]*Functional Imaging Unit, Dept. of Clinical Physiology, Nuclear Medicine and PET, Rigshospitalet, Copenhagen, Denmark*

[2] *Clinical Neurosciences, Clinical and Experimental Sciences, Faculty of Medicine, University of Southampton, United Kingdom*

[3]*Dept. of Neurology, Rigshospitalet, Glostrup, Denmark*

[4]*Institute of Clinical Medicine, Faculty of Health and Medical Science, Copenhagen University, Denmark*

Corresponding author

Stig P. Cramer, MD, PhD

Functional Imaging Unit, Dept. of Clinical Physiology, Nuclear Medicine and PET,

Rigshospitalet, Ndr. Ringvej 57, DK-2600 Glostrup, Denmark

Tel: +45 3863 4624

E-mail: stig.praestekjaer.cramer@regionh.dk

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process which may lead to differences between this version and the Version of Record. Please cite this article as an 'Accepted Article', doi: 10.1002/ana.25219

Word count
Title: 177 characters
Running head: 50 characters

Abstract: 248
Introduction: 481
Discussion: 1639
Body of the manuscript: 5121

Number of figures: 6
Color figures: 0
Tables: 2

Abstract

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

Methods: 35 RRMS patients starting on fingolimod or natalizumab, drugs with a common effect of decreasing lymphocyte influx into the CNS, were scanned with DCE-MRI at 3T prior to treatment and at three and six-months post-treatment. We calculated the influx constant K_i, a measure of BBB permeability, using the Patlak model. Sub-optimal treatment response was defined as loss of no evidence of disease activity (NEDA-3) status after two years of treatment.

Results: Subjects with loss of NEDA status at 2 years had a 51% higher mean K_i in normal-appearing white matter (NAWM) measured after six months of treatment, compared to subjects with maintained NEDA status (mean difference 0.06 (CI 0.02-0.09) ml/100g/min; p=0.002). K_i in NAWM at 6 months was a good predictor of loss of NEDA status at two years (AUC 0.84, CI 0.70-0.99; p=0.003) and a value above 0.136 ml/100/g/min yielded an odds ratio of 12.4 for sub-optimal 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 sub-optimal 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.

Keywords. MRI; DCE MRI; perfusion MRI; blood-brain barrier; disease-modifying therapy; multiple sclerosis

Introduction

A large number of immunomodulatory disease-modifying therapies (DMTs) are now available for RRMS. Their main objective is a reduction in the number and severity of relapses, occurrence of new or enlarging lesions on 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 $\alpha 4\beta 1$ integrin receptor, which mediates lymphocyte adherence to the endothelium, hereby directly suppressing lymphocyte passage across the blood-brain barrier (BBB).[2] Fingolimod is an agonist of the sphingosine-1 receptor, inducing receptor internalization and thereby trapping encephalitogenic lymphocytes in lymph nodes[3] preventing them from migrating into the CNS. Hence, although the mechanism of action of these two drugs is different, their final effect is the same i.e. a reduction of the absolute number of lymphocytes trafficking across the BBB, as demonstrated by an equivalent reduction in CD4 lymphocyte counts in the CSF.[4] Both treatments have been shown to be highly efficacious in reducing relapse rates by 54-68%, reducing occurrence of new T2 lesions on MRI as well as the number of visibly contrast-enhancing lesions.[5–7] Despite the high overall efficacy, the treatment response is highly heterogeneous and a subset of patients still experience disease activity.[8] In order 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 three domains) has been proposed as a treatment goal.[9–11] Evaluating NEDA status after two years of DMT may be a reasonable approach, since it holds a positive predictive value of 78.3% for no progression at 7 years, with only minor improvement for re-evaluation at years 3-5.[11] However, early detection of sub-optimal treatment response is becoming increasingly important, both in the context of the increasing number of available therapies[5,12,13] and due to the fact that DMTs seem to have their best effect in the early stages of disease,[14] but no current method or clinical variable exists that is

able to perform such stratification.[8,15] We have previously reported that BBB permeability in multiple sclerosis (MS) normal-appearing white matter (NAWM), measured as the influx constant K_i 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.[16] K_i in NAWM correlates with biomarkers of immune cell trafficking in the cerebrospinal fluid, and predicts conversion from optic neuritis to MS two years after onset.[17] Hence, we hypothesize that K_i can stratify MS patients according to DMT response, here defined as loss of NEDA-3 status at one and two years of second-line treatment. Furthermore, we aim to characterize the mechanistic relationship between K_i and cellular traffic, in the setting of treatments whose common end result is a reduction in lymphocyte traffic across the BBB.

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, 3) Age 18-59 years. Exclusion criteria were: 1) Other concurring disease, 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 three months post-treatment. Twenty-nine patients participated in the six-month post-treatment MRI. After study completion, two subjects were excluded, the first due to the occurrence of anti-natalizumab antibodies, which resulted in treatment cessation after five months of treatment, and the second due to suspected side-effects to fingolimod in the form of macular oedema, which resulted in treatment cessation

after eight months. Follow-up MRI scans were performed as close as possible to the three (mean 97 days; standard deviation (SD) 13 days) and six months time points (mean 189 days; SD 17 days) post-treatment using the therapy initiation day as reference and consisted of axial T2, axial FLAIR and axial post contrast T1 of the cerebrum as well as the DCE-MRI (see below for sequence parameters). Spinal cord assessment was performed at baseline, but not at the 3 and 6 months follow-up scans. We recorded any use of methylprednisolone during the course of the study, with the intention to postpone any three or six-month follow-up scan by two months after completion of steroid treatment. Only one subject was treated with intravenous methylprednisolone during the first six months of the study. This occurred four months after treatment initiation, due to a major relapse 65 days before the planned six-month scan, obviating the need for postponing the scan. Clinical data was obtained from hospital records two 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 EDSS at one and two years after treatment initiation. All subjects had regular clinical follow-up visits three, six, twelve, eighteen and twenty-four months post-treatment as part of standard clinical practice. Anti-natalizumab antibodies were measured at three, six, nine, and 12 months. Collection of clinical data was performed by an experienced MS clinician who was blinded to the DCE-MRI results (another researcher analysed 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 one or two years of treatment were included in the one and two-year analyses.

Outcome Measures

We used the following definitions. Relapse was defined as the appearance of new neurological symptoms or signs that lasted more than 24 hours in the absence of concurrent fever or illness.[18]

The treating physician recorded relapses at the face-to-face visits at three, six, 12, 18 and 24 months. Progression was defined as an EDSS score increase of one or more points recorded at a biannual clinical visit that was sustained at the subsequent clinical visit six months later.[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 hyperintense 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 three 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 three NEDA subdomains were 1) a new T_2 lesion at six months (this subject had another new T_2 lesion at one 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 six months 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.

Dynamic contrast enhanced (DCE) MRI. MRI was performed on a 3T MR unit (Philips Achieva) 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 ms, echo time = 1.9 ms, centric phase ordering, parallel imaging factor 2, acquired matrix 96 x 61, acquired voxel-size 2.40 x 2.98 x 8 mm^3 (interpolated to 0.90 x 0.89 x 8 mm^3), field of view 230 x 182 mm^2, five slices, slice thickness 8 mm. Data for an initial measurement of relaxation time (T1) and equilibrium magnetization (M0) were generated using a series of saturation time delays from 120 ms to 10 s, covering the same slices as imaged during the bolus passage. The dynamic

sequence used a saturation time delay of 120 ms, giving a time resolution of 1.25 s, and 750 time points, corresponding to a total sampling duration of 15.7 min. The automatic bolus injection (Spectris, Medrad; USA) with speed 3 ml/s followed by 20 ml saline was started after the 10th time point. The dose of contrast agent (Gadobutrol 1 mmol/ml) was 0.045 mmol/kg bodyweight. We acquired a separate slice at the level of the internal carotid artery (ICA) to obtain an arterial input function with minimal partial volume for every single subject. The remaining four DCE slices were used for defining regions of interest and subsequent estimation tissue pharmacokinetic values. To achieve a full clinical dose of Gadobutrol (0.1 ml/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 post-contrast T1 sequence.

MRI sequences and regions of interest. We used an axial T2- weighted MRI sequence [five slices, echo time = 100 ms, repetition time = 3000 ms, acquired voxel-size 0.57 x 0.76 x 8 mm^3 (interpolated to 0.45 x 0.45 x 8 mm^3), field of view = 230 x 119 mm^2] with same orientation and slice thickness (8 mm) as our DCE-MRI sequence, in order to manually draw regions of interest in the periventricular NAWM, and in the normal-appearing thalamic grey matter in both hemispheres, avoiding inclusion of, or proximity to, any MS lesions or diffusely abnormal white matter, as previously described in detail.[16] Four regions of interest were placed in periventricular NAWM (two in the vicinity of the frontal ventricular horns, one each hemisphere and two in the vicinity of the posterior horn, one in each hemisphere). Examples of region of interest placement on anatomical images and corresponding K_i maps from two subjects can be seen in Fig 2. T2 lesions counts were performed by an experienced neuroradiologist using an axial T2 fluid attenuation inversion recovery (FLAIR) sequence [35 slices, echo time = 125 ms, repetition time = 11 000 ms, acquired voxel-size 0.65 x 0.99 x 3.5 mm^3 (interpolated to 0.45 x 0.45 x 3.5 mm^3), field of view = 230 x 119 mm^2, slice thickness of 3.5 mm]. Regions of interest were placed a

minimum of 10 mm from any MS lesion or CSF-containing structures. In the presence of contrast-enhancing lesions on a post contrast axial T1 weighted spin echo sequence [44 slices, echo time = 10 ms, repetition time = 600 ms, acquired voxel-size 0.94 x 1.25 x 3 mm^3 (interpolated to 0.94 x 0.94 x 3 mm^3), field of view = 240 x 240 mm^2, slice thickness of 3 mm] we took care not to include the nearest 30 mm of non-enhancing tissue. Our four DCE slices were placed with exactly the same angulation and anatomical position as the previous scan (evaluated for every single scan). We ensured consistent positioning and size of our regions of interest across different study time points by visual alignment with the previous scan.

Permeability estimation. The DCE MRI data was analysed with a semi-automated procedure[22] using in-house Matlab-based software. The DCE time series was converted to units of contrast agent concentration using T_1 and M_0, as determined from the multiple saturation delay data, and a contrast agent relaxivity of 4 $sec^{-1}mM^{-1}$. 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[23] ad modum Van Osch.[24] 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 (e.g four regions of NAWM was drawn, of which the median was used to represent NAWM). Every subject was represented by one value calculated as a mean of the tissue specific regions of interest, as previously described.[16,17,25] Tissue concentration-time curves were evaluated using a combination of model-free deconvolution and a Patlak model, as described in previous work.[26] Permeability values, measured as K_i (full blood) relates to K^{trans} (plasma) by $K_i=K^{trans}/(1\text{-Hct})$. A fixed value of Hct = 0.45 was used throughout the study. Values of K_i are reported as mL/100g/min assuming brain tissue density of 1 g/ml.[27]

Statistics. Histograms, probability plots and modified Kolmogorov-Smirnov (Lillefors) testing was used to analyse continuous variables for standard normal distribution fit.[28,29] If the data were found to follow a normal distribution two-tailed Student's 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 K_i and MS clinical parameters. A one-way repeated measures 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 K_i to predict sub-optimal 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 when there were more than two possible outcomes. A p-value lower than 0.05 allowed rejection of the null hypothesis. All analyses were performed in SPSS version 23.

Multiple comparisons The *a priori* hypothesis was that K_i after treatment initiation predicts sub-optimal treatment effect, and have thus investigated the performance of four different variables (K_i in NAWM and thalamus at three and six months). Applying a Bonferroni correction but taking the correlation coefficient (average CC=0.49) between the measured variables into account by way of the Dubey & Armitage-Parmar approach,[30,31] 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 (β=-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 (β=-0.71, p=0.00008) and days since last relapse (β=-0.48, p=0.005) predicted baseline K_i 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 K_i was predicted by methylprednisolone treatment 2 months prior to baseline (β=-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 Fig 3.

Treatment effect Between subject receiving natalizumab and fingolimod there was no difference in mean K_i pre-treatment (NAWM: mean difference 0.008 ml/100g/min; CI −0.05-0.06; p=0.78, thalamus: mean difference 0.01 ml/100g/min; CI −0.04-0.06; p=0.65) and six months post-treatment (NAWM: mean difference 0.004 ml/100g/min; CI −0.04-0.05; p=0.84, thalamus: mean difference 0.0002 ml/100g/min; CI −0.05-0.05; p=0.99). However, at three months post-treatment K_i in NAWM (mean difference 0.06 ml/100g/min; CI 0.02-0.10; p=0.002) and thalamus (mean difference 0.04 ml/100g/min; CI 0.01-0.08; p=0.011; both Student's t tests) was higher in the natalizumab treated patients, possibly reflecting a clinical selection bias favouring treatment of patients with highly active disease with natalizumab, as previously seen.[32,33] Of natalizumab treated subjects 3/11 had a relapse during the first six months of treatment (occurred seven, eight, and 133 days post-treatment) as opposed to 1/24 fingolimod treated subjects (occurred 20 days post-treatment); possibly reflecting the same bias. A one-way repeated measures ANOVA analysis with K_i in NAWM pre-treatment and six months post-treatment 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), see 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 one year of second-line treatment 12 out of 35 subjects (34%) lost NEDA-3 status. After two years this increased to 15 out of 35 (43%). Five out of 11 (45%) natalizumab treated subjects and 10 out of 24 (42%) of fingolimod treated subjects had lost NEDA status at two years. Of the 15 subjects who lost NEDA status at 2 years, four subjects had activity in all three NEDA subdomains (relapse(s), new MRI activity, and EDDS increase), three subjects had relapse(s) and EDSS increase, one subject had new MRI activity and EDSS increase, four subjects relapse(s) only, two subjects EDSS increase only, and one subject MRI activity only. Baseline demographics, clinical characteristics and K_i values according to NEDA status at two years are shown in Table 1. Three subjects experienced a relapse shortly after starting treatment (seven, eight and 20 days after treatment initiation), but per protocol these were disregarded. Subjects who lost NEDA status at two years had a 51% higher K_i in NAWM at six months post-treatment (mean difference 0.06 ml/100g/min; CI 0.02-0.09; p=0.002) and a 78% higher annual relapse rate (ARR) one-year pre-treatment (mean difference 0.93; CI 0.38-1.5; p=0.002; all Student's t-tests), when compared to subjects who maintained NEDA status, see Table 1 and Fig 5. K_i at baseline and three months in NAWM and thalami were non-significant between NEDA groups (NAWM baseline: mean difference 0.013 ml/100g/min, CI: −0.04-0.07, p=0.62; thalamus baseline: mean difference 0.02 ml/100g/min, CI: −0.03-0.07, p=0.39; NAWM three months: mean difference 0.016 ml/100g/min, CI: −0.03-0.06, p=0.45; thalamus three months: mean difference 0.02 ml/100g/min, CI: −0.02-0.06, p=0.25), while thalamic K_i at six months showed an insignificant trend for higher values (mean difference 0.043 ml/100g/min, CI: 0.002-0.09, p=0.040) in the loss of NEDA status group. In subjects who lost NEDA status at one year only ARR one-year pre-treatment was significantly higher (mean difference 0.99, CI: 0.42-1.56, p=0.001). A receiver operating characteristics (ROC) curve with

loss of NEDA at 2 years as outcome showed that K_i in NAWM at six months was a good predictor of loss of NEDA status at two years, with an area under the curve (AUC) of 0.84 (CI 0.70 – 0.99), p=0.003, see Fig 6. The optimal threshold, defined as the value that provided the highest added sensitivity and specificity[34] of K_i in NAWM for detecting loss of NEDA was 0.136 ml/100g/min providing a sensitivity of 73% and specificity of 82%. More than one annual relapse one-year pre-treatment predicted loss of NEDA (AUC 0.79; CI 0.64 – 0.94, p=0.004) with a sensitivity of 87% and specificity of 65%. Univariate logistic regression analysis showed that K_i in NAWM at 6 months was associated with loss of NEDA at 2 years (an increase of one standard deviation (0.05 ml/100g/min) yielded an odds ratio of 10.; CI 1.4-74; p=0.02), as was number of annual relapses one-year pre-treatment (OR 9.2; CI 1.8 – 48; p=0.009), but not presence of active T2 lesions at 6 months, K_i in NAWM at 3 months, K_i 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 K_i in NAWM > 0.136 ml/100g/min yielded an odds ratio of 12.4 for loss of NEDA at two years while >1 annual relapses one-year pre-treatment was now insignificant see 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 K_i was measured. Excluding these two subjects from the ROC curve analysis of K_i in NAWM at 6 months with NEDA at 2 years as outcome, only caused minor changes to the results (AUC 0.84 (CI 0.70-0.99), p=0.003, sensitivity 73%, specificity 81%).

DCE-MRI versus conventional contrast imaging

10 out of 35 subjects (~29%) had one or more contrast-enhancing lesions on baseline MRI, and while these subjects had higher mean values of K_i at baseline (NAWM 0.15 ml/100g/min; thalami 0.15 ml/100g/min), the difference was not significant when compared to subjects without contrast-enhancing lesions (NAWM 0.14 ml/100g/min; thalami 0.14 ml/100g/min),

mean difference NAWM 0.01 ml/100g/min (CI –0.05-0.07), and thalamus 0.01 ml/100g/min (CI –0.03-0.05). Only two subjects had contrast-enhancing lesions on the three months follow-up MRI and no subjects showed contrast-enhancement at the six months 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 (Qalb), perhaps suggesting that K_i may be a surrogate marker of cellular influx.[17] This study addresses this issue, since 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 six month but not three month K_i predicted NEDA after two 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 three months after initiation of treatment. In those losing NEDA, one can hypothesise on-going 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, whilst having no effect on the permeability to albumin.[35]

We have previously reported that a K_i above 0.13 ml/100g/min identifies optic neuritis (ON) subjects with high risk of conversion to RRMS, adding significant value compared to using T2 lesions alone.[17] It is very interesting to note that the ROC threshold for further disease activity is identical in CIS[17] versus established RRMS. This solute BBB permeability threshold could be acting as a reproducible surrogate marker for a BBB state associated with active disease.

Prediction of two year NEDA status

We also report the novel finding that a single measurement of BBB permeability in NAWM performed six months after initiation of natalizumab or fingolimod is capable of predicting loss of NEDA status within the first two years of treatment. Since NEDA at two years has recently been shown to predict disability progression as measured by EDSS at seven years nearly as well as NEDA at five years,[11] 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 above 0.136 ml/100g/min predicts loss of NEDA at two years (odds ratio [OR] of 11.6). For comparison, two or more contrast enhancing lesions in the first year of treatment with interferon beat-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.[36]

Presence of visibly contrast-enhancing lesions was not a significant determinant of K_i in NAWM or thalamus at neither 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 carry-over from the prognostic effect of contrast-enhancing lesions or a result of spill over of contrast agent from enhancing lesions into the surrounding NAWM. Assuming a textbook value of water diffusion in brain tissue of 1.0×10^{-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} $m^2/15*60 = 0.95$ mm. Thus, it is highly unlikely that water from a gadolinium enhancing lesion should diffuse into our NAWM ROI's. However, one study found contrast-enhancing lesions in the first year of natalizumab treatment to predict future disease progression.[38] Indeed, in our cohort, contrast-enhancing lesions were only seen in two subjects at three months and none were observed at six months or at one year. Despite this, K_i at six months predicted NEDA at two 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, as well as diffuse vessel leakage.[39–41] DCE-MRI has the additional advantage of using a lower dose of gadolinium contrast, compared to conventional contrast MR imaging.

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 six-month K_i and days since last relapse or baseline contrast-enhancing lesion count; we only observe one relapse in close proximity to the six-month scan. This dispels the possibility that the predictive effect of the higher K_i at six months on two year NEDA is a throwback to higher baseline disease activity.

Possible reasons for treatment failure

We found that K_i above 0.136 ml/100g/min predicts sub-optimal natalizumab or fingolimod treatment response with a sensitivity of 73% and specificity of 81%. Possible reasons for

treatment failure could be: (1) lack of compliance (2) neutralizing antibodies (3) uncoupling of the disease process from the drug's mechanism of action (4) high intrinsic disease activity. Lack of compliance and neutralizing antibodies were excluded in this study since 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 which 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 GLM model, we find a trend towards an interaction effect of time and baseline methylprednisolone treatment, indicating a treatment effect between paired baseline and six-month K_i only if baseline methylprednisolone is accounted for. This is indicative of a cumulative "K_i response" to both types of treatment, i.e. 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 indeed 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 sub-optimal and optimal treatment responses in the same individuals.

Proportion of NEDA

In this study, the proportion of subjects with loss of NEDA-3 status was 34% during the first year and 43% during the second year. One natalizumab study reported loss of NEDA-3 status at 2 years in 38% of subjects, but that study used less stringent MRI criteria not including enlarging

T2 lesions.[42] In the AFFIRM trial, 63% had lost NEDA status after 2 years of natalizumab treatment, using the same NEDA-3 criteria as in our study.[19] A head-to-head comparison of NEDA-3 in natalizumab vs. fingolimod treatment showed loss of NEDA status at 2 years in 30% and 77% respectively.[43] 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 favoured 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 Gd-DTPA administration in 27 RRMS patients after 24 weeks on natalizumab treatment, but found no effect of treatment on signal change in NAWM[44] when compared to 13 patients receiving placebo. No clinical parameters, such as recent methylprednisolone treatment, relapses and individual treatment effect were taken into account; variables that we have shown govern K_i. This emphasizes the importance of including clinical covariates when characterising changes in K_i over time. Solute permeability across the BBB, which is mainly governed by diffusion,[45,46] 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 547 Da highly hydrophilic molecule.[47] 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*.[45,46,48] 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 timepoint, indicates 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 six 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 sub-optimal treatment response in RRMS, and a personalised medicine approach to treatment, limitations being the long scan time (15 minutes). These results and the proposed thresholds require validation in larger studies.

Acknowledgements

We would like to thank radiographers Bente Sonne Møller and Karina Elin Segers at Dept. of Diagnostics, Glostrup Hospital for scanning assistance and the MD consultants Alex Heick, Rikke Jensen, Houry Hassanpour, and Anna Tsakiri at the MS Clinic, Dept. of Neurology, Rigshospitalet, Glostrup for referring patients for the project. Many thanks to Ulrich Lindberg for Matlab help. We would like to express our gratitude to the patients for participating. This work was supported by The Research Foundation of the Capital Region of Denmark, Foundation for Health Research [grant number R129-A4197]; The Danish Council for Independent Research [grant number DFF-6110-00061] Rigshospitalets forskningspuljer [grant number R113-A4596-B2759]; The Danish Multiple Sclerosis Society [grant number 14588] and Biogen Idec [grant number GLO-01-2012].

Author contributions

Study concept and design: SPC, JF, HBWL

Data acquisition and analysis: SPC, HJS, JF, HBWL

Drafting the text and figures: SPC, HJS, AV, IG, JF, HBWL

Conflict of Interest Statement

SPC has received research funding and travel funding from Biogen Idec. JF has served on scientific advisory boards for and received funding for travel related to these activities as well as speaker honoraria from Biogen Idec. HBWL 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. HJS, IG and AV report no conflicts of interest for the present study.

References

1. Río 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. [Internet]. Nature 1992;356(6364):63–6.[cited 2017 May 15] Available from: http://www.nature.com/doifinder/10.1038/356063a0
3. Matloubian M, Lo CG, Cinamon G, et al. Lymphocyte egress from thymus and peripheral lymphoid organs is dependent on S1P receptor 1. [Internet]. Nature 2004;427(6972):355–60.[cited 2017 May 15] Available from: http://www.nature.com/doifinder/10.1038/nature02284
4. Kowarik MC, Pellkofer HL, Cepok S, et al. Differential effects of fingolimod (FTY720) on immune cells in the CSF and blood of patients with MS. [Internet]. Neurology 2011;76(14):1214–21.[cited 2017 May 15] Available from: http://www.neurology.org/cgi/doi/10.1212/WNL.0b013e3182143564
5. Polman CH, O'Connor PW, Havrdova E, et al. A randomized, placebo-controlled trial of natalizumab for relapsing multiple sclerosis. 2006.
6. Kappos L, Radue E-W, O'Connor P, et al. A placebo-controlled trial of oral fingolimod in relapsing multiple sclerosis. 2010.
7. Cohen J a, Barkhof F, Comi G, et al. Oral fingolimod or intramuscular interferon for relapsing multiple sclerosis. N. Engl. J. Med. 2010;362:402–415.
8. Río J, Comabella M, Montalban X. Predicting responders to therapies for multiple sclerosis. [Internet]. Nat. Rev. Neurol. 2009;5(10):553–60.[cited 2016 Nov 17] Available from: http://www.nature.com/doifinder/10.1038/nrneurol.2009.139
9. Lublin FD. Disease activity free status in MS [Internet]. Mult. Scler. Relat. Disord. 2012;1(1):6–7.Available from: http://dx.doi.org/10.1016/j.msard.2011.08.001
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(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. [Internet]. JAMA Neurol. 2015;72(2):152–8.[cited 2016 Apr 20] Available from: http://archneur.jamanetwork.com/article.aspx?doi=10.1001/jamaneurol.2014.3537
12. Kappos L, Havrdova E, Giovannoni G, et al. No evidence of disease activity in patients receiving daclizumab versus intramuscular interferon beta-1a for relapsing-remitting multiple sclerosis in the DECIDE study. [Internet]. Mult. Scler. 2016;21(11_suppl):1352458516683266.[cited 2017 Mar 8] Available from: http://journals.sagepub.com/doi/10.1177/1352458516683266
13. Kalincik T, Brown JWL, Robertson N, et al. Treatment effectiveness of alemtuzumab compared with natalizumab, fingolimod, and interferon beta in relapsing-remitting multiple sclerosis: a cohort study [Internet]. Lancet Neurol. 2017;4422(17):1–11.Available from: www.thelancet.com/neurology
14. Freedman MS, Abdoli M. Evaluating response to disease-modifying therapy in relapsing

multiple sclerosis. [Internet]. Expert Rev. Neurother. 2015;15(4):407–23.[cited 2017 Mar 2] Available from: http://www.ncbi.nlm.nih.gov/pubmed/25764966

15. Río J, Nos C, Tintoré M, et al. Defining the response to interferon-β in relapsing-remitting multiple sclerosis patients. Ann. Neurol. 2006;59(2):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 [Internet]. NeuroImage Clin. 2014;4:182–189.Available from: http://dx.doi.org/10.1016/j.nicl.2013.12.001

17. Cramer SP, Modvig S, Simonsen HJ, et al. Permeability of the blood-brain barrier predicts conversion from optic neuritis to multiple sclerosis [Internet]. Brain 2015;138(9):2571–2583.Available from: http://www.brain.oxfordjournals.org/lookup/doi/10.1093/brain/awv203

18. Schumacker G a, Beebe G, Kibler RF, et al. Problems of Experimental 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(1):552–568.

19. Havrdova E, Galetta S, Hutchinson M, et al. Effect of natalizumab on clinical and radiological disease activity in multiple sclerosis: a retrospective analysis of the Natalizumab Safety and Efficacy in Relapsing-Remitting Multiple Sclerosis (AFFIRM) study [Internet]. Lancet. Neurol. 2009;8(3):254–260.Available from: http://dx.doi.org/10.1016/S1474-4422(09)70021-3

20. Stangel M, Penner IK, Kallmann BA, et al. Towards the implementation of "no evidence of disease activity" in multiple sclerosis treatment: the multiple sclerosis decision model. [Internet]. Ther. Adv. Neurol. Disord. 2015;8(1):3–13.Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=4286940&tool=pmcentrez&rendertype=abstract

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 [Internet]. Nat. Rev. Neurol. 2015;11(August):1–12.Available from: http://www.nature.com/doifinder/10.1038/nrneurol.2015.106

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 T 1-weighted perfusion imaging and limitations of the multiplicative rescaling approach. Magn. Reson. Med. 2009;62:1055–1059.

24. Van Osch MJP, Vonken EJP a, Bakker CJG, Viergever M a. Correcting partial volume artifacts of the arterial input function in quantitative cerebral perfusion MRI. Magn. Reson. Med. 2001;45(March 2000):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 [Internet]. J. Cereb. Blood Flow Metab. Off. J. Int. Soc. Cereb. Blood Flow Metab. 2014;34(10):1655–1665.Available from: http://www.ncbi.nlm.nih.gov/pubmed/25074746

26. Larsson HBW, Courivaud F, Rostrup E, Hansen AE. Measurement of brain perfusion,

blood volume, and blood-brain barrier permeability, 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. [Internet]. Acta Neurol. Scand. 1970;46(1):85–92.[cited 2017 Apr 21] Available from: http://www.ncbi.nlm.nih.gov/pubmed/4983875

28. Lilliefors HW. On the Kolmogorov-Smirnov Test for Normality with Mean and Variance Unknown [Internet]. J. Am. Stat. Assoc. 1967;62(318):399.[cited 2017 May 2] Available from: http://www.jstor.org/stable/2283970?origin=crossref

29. Dallal GE, Wilkinson L. An Analytic Approximation to the Distribution of Lilliefors's Test Statistic for Normality [Internet]. Am. Stat. 1986;40(4):294.[cited 2017 May 2] Available from: http://www.jstor.org/stable/2684607?origin=crossref

30. Sankoh AJ, Huque MF, Dubey SD. Some comments on frequently used multiple endpoint adjustment methods in clinical trials. Stat. Med. 1997;16(22):2529–2542.

31. Blakesley RE, Mazumdar S, Dew MA, et al. Comparisons of methods for multiple hypothesis testing inNeuropsychological Research. Neuropsychology 2009;23(2):255–264.

32. Barbin L, Rousseau C, Jousset N, et al. Comparative efficacy of fingolimod vs natalizumab: A French multicenter observational study. [Internet]. Neurology 2016;86(8):771–8.Available from: http://www.ncbi.nlm.nih.gov/pubmed/26826205

33. Kalincik T, Horakova D, Spelman T, et al. Switch to natalizumab versus fingolimod in active relapsing-remitting multiple sclerosis. Ann. Neurol. 2015;77(3):425–435.

34. Zou KH, O'Malley AJ, Mauri L. Receiver-operating characteristic analysis for evaluating diagnostic tests and predictive models. Circulation 2007;115(5):654–657.

35. Prat A, Biernacki K, Antel JP. Th1 and Th2 lymphocyte migration across the human BBB is specifically regulated by interferon β and copolymer-1 [Internet]. J. Autoimmun. 2005;24(2):119–124.[cited 2017 Apr 24] Available from: http://www.ncbi.nlm.nih.gov/pubmed/15829404

36. Bermel RA, You X, Foulds P, et al. Predictors of long-term outcome in multiple sclerosis patients treated with interferon beta. Ann. Neurol. 2013;73(1):95–103.

37. Le Bihan D, Iima M. Diffusion magnetic resonance imaging: What water tells us about biological tissues. PLoS Biol. 2015;13(7):1–13.

38. Raffel J, Gafson AR, Dahdaleh S, et al. Inflammatory Activity on Natalizumab Predicts Short-Term but Not Long-Term Disability in Multiple Sclerosis [Internet]. PLoS One 2017;12(1):e0169546.Available from: http://dx.plos.org/10.1371/journal.pone.0169546

39. Ludwin SK. The pathogenesis of multiple sclerosis: relating human pathology to experimental studies. J. Neuropathol. Exp. Neurol. 2006;65(4):305–318.

40. Moll NM, Rietsch AM, Thomas S, et al. Multiple sclerosis normal-appearing white matter: Pathology-imaging correlations. Ann. Neurol. 2011;70(5):764–773.

41. Plumb J, McQuaid S, Mirakhur M, Kirk J. Abnormal endothelial tight junctions in active lesions and normal-appearing white matter 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 [Internet]. J. Neurol. Sci. 2016;364:145–147.Available from: http://dx.doi.org/10.1016/j.jns.2016.03.025

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. [Internet]. Mult. Scler. 2016;1315–1326.Available from: http://www.ncbi.nlm.nih.gov/pubmed/27230789

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(1):5–11.

46. Varatharaj A, Galea I. The blood-brain barrier in systemic inflammation. [Internet]. Brain. Behav. Immun. 2017;60:1–12.[cited 2016 May 20] Available from: http://linkinghub.elsevier.com/retrieve/pii/S0889159116300551

47. Saremi F. Perfusion Imaging in Clinical Practice : A Multimodality Approach to Tissue Perfusion Analysis. 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. [Internet]. Fluids Barriers CNS 2011;8(1):4.Available from: http://www.fluidsbarrierscns.com/content/8/1/4

Table and Figure captions:

Table 1 **Demographical, clinical and K_i values according to NEDA status two years post second-line treatment.** Values are mean +/- standard deviation. K_i at six months and number of relapses before treatment start were significantly higher in subjects with loss of NEDA status at 2 years. K_i in thalamus at six months showed a trend for higher values but was non-significant. * Only entered for subjects who received steroids treatment within the last two months. a) Student's T-test; b) Student's T-test on log-transformed data; c) Chi square; d) Chi square with first-line treatment yes/no. Abbreviations: NAWM = Normal appearing white matter, THAL = Thalamus, Gd+ = Gadolinium enhancing lesion(s)

Table 2 **Results of stepwise multivariate logistic regression with loss of NEDA status within the first 2 years of second-line treatment as outcome variable.** 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 one year before treatment start showed a trend but was non-significant. (a) From ROC curve analysis (b) Predicted loss of NEDA status. Abbreviations: NAWM = Normal appearing white matter, CI = Confidence interval

Figure 1 **Subject inclusion procedure.**

Figure 2 **Arterial input function in arbitrary signal units (A - left) and concentration (A right), and region of interest placement**. Examples from two subjects, left column from a subject with maintained NEDA status after two years of treatment (A1, B1, C1, and D1) and right column from a subjects with loss of NEDA status after two years of treatment (A2, B2, C2, and D2). Region of interest placement represents two examples in normal appearing white matter (B and C) and one in thalamus (D) with their corresponding Patlak plots at six months post-treatment.

Figure 3 **Baseline permeability in normal appearing white matter (NAWM) for all subjects with a baseline scan (n=45) according to current treatment and recent relapse.** SPSS 23 standard setup for boxplot presentation was used (black line represents the median, boxes represent the interquartile range (IQR: data between the 25 and 75% quartiles). Whiskers represent 1.5 times the IQR. Outliers (open circles) are defined as data points outside 1.5 x IQR). One subject was treated with pulsed steroids (50 mg every first three days/month), and thus received methylprednisolone treatment in spite of not having had a recent relapse. Abbreviations: IFN-Beta = Interferon Beta. GA = Glatiramer acetate.

Figure 4 **K_i in periventricular normal-appearing white matter (NAWM) during the course of second line treatment for subjects who completed all three visits (n=27).** Coloured bold lines represent mean K_i according to which treatment the subjects received *prior* to second-line treatment (blue=no prior treatment, green=interferon beta (IFN-Beta) or glatiramer acetate (GA), brown=methylprednisolone within the last two months). Baseline scan was conducted shortly prior to second-line treatment initiation and follow-up scans were conducted at three and six months after second-line treatment. Error bars represent +/- 1 SEM.

Figure 5 **K_i in periventricular normal-appearing white matter (NAWM; top row) and thalamus (bottom row) pre- natalizumab or fingolimod treatment (baseline) and three and six months post-treatment.** Horizontal dotted line represents optimal threshold for loss of NEDA status from the ROC curve analysis. Blue line represents mean K_i in subjects with maintained no-evidence of disease-activity (NEDA) status at two years and green line represents mean K_i in subjects with lost NEDA status. Error bars represent +/- 1 SEM.

Figure 6 **Result of receiver operator characteristic (ROC) curve analysis with loss of no evidence of disease activity (NEDA) status as outcome variable**. Blue= K_i in NAWM at 6 months; Green = K_i in thalamus at 6 months; Orange= Annual relapse rate one-year prior to treatment start; Red= New active T2 lesions at 6 months. Abbreviations: AUC = Area under the curve.

	NEDA status at 2 years		
	Lost (n=15)	**Maintained (n=20)**	**P value**
Age (years)	36 (8.2)	43.1 (9.9)	0.03 [(a)]
Gender (number of women)	9 (60%)	14 (70%)	0.72 [(c)]
EDSS score baseline	2.5 (1.6)	3.2 (1.4)	0.17 [(a)]
Disease duration (years)	4.7 (3.7)	8.1 (6.8)	0.09 [(a)]
Number of relapses one year before treatment start	2.1 (0.9)	1.2 (0.7)	**0.002** [(a)]
Last relapse onset (days)	150 (124)	137 (110)	0.75 [(a)]
Relapse within three months from baseline	8 (53%)	12 (60%)	0.74 [(c)]
Baseline treatment			1.00 [(d)]
None	3 (20%)	5 (25%)	
Interferon-β	10 (67%)	13 (65%)	
Glatiramer acetate	2 (13%)	2 (10%)	
Methylprednisolone < two months	4 (27%)	4 (20%)	0.70 [(c)]
Days since treatment end *	27 (23)	39 (29)	0.80 [(a)]
Baseline MRI			
T_2 lesion count	19.1 (12.7)	14.7 (8.5)	0.56 [(b)]
T_2 lesion volume (mm^3)	14.5 (15.2)	8.3 (3.5)	0.30 [(b)]
≥1 Gd+ lesion	5 (33%)	5 (25%)	1.00 [(c)]
Second-line treatment type (natalizumab)	5 (33%)	6 (30%)	1.00 [(c)]
K_i 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)]
Three months (n=35)	0.144 (0.049)	0.129 (0.062)	0.45 [(a)]
Size (voxels)	152 (92)	161 (83)	0.76 [(a)]
Six months (n=28)	0.166 (0.059)	0.110 (0.029)	**0.002** [(a)]
Size (voxels)	170 (99)	179 (86)	0.77 [(a)]
K_i 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)]
Three months (n=35)	0.143 (0.042)	0.125 (0.054)	0.25 [(a)]
Size (voxels)	115 (43)	130 (46)	0.33 [(a)]
Six months (n=28)	0.165 (0.069)	0.122 (0.037)	0.04 [(a)]
Size (voxels)	143 (50)	136 (47)	0.67 [(a)]

Table 1 **Demographical, clinical and K_i values according to NEDA status two years post second-line treatment.** Values are mean +/- standard deviation. K_i at six months and number of relapses before treatment start were significantly higher in subjects with loss of NEDA status at 2 years. K_i in thalamus at six months showed a trend for higher values but was non-significant. * Only entered for subjects who received steroids treatment within the last two months. a) Student's T-test; b) Student's T-test on log-transformed data; c) Chi square; d) Chi square with first-line treatment yes/no. Abbreviations: NAWM = Normal appearing white matter, THAL = Thalamus, Gd+ = Gadolinium enhancing lesion(s)

Variable	Optimal cut-off[a]	Predicted[b], *n*	Observed, *n* (% correct)	P value	Odds ratio	95% CI
K_i in NAWM six months post treatment	> 0.136 ml/100g/min	8 positives	11 (73%)	0.007	12.4	2 – 77
		14 negatives	17 (82%)			
K_i in the thalamus six months post treatment	> 0.124 ml/100g/min	9 positives	11 (82%)	0.10	-	-
		11 negatives	17 (65%)			
Number of relapses one year before treatment start	> 1	13 positives	15 (87%)	0.07	-	-
		13 negatives	20 (65%)			
Baseline T_2 lesion count	> 13	10 positives	15 (67%)	0.94	-	-
		12 negatives	20 (60%)			
Active T_2 lesions at 6 months	> 0	2 positives	11 (18%)	0.06	-	-
		18 negatives	18 (100%)			

Table 2 **Results of stepwise multivariate logistic regression with loss of NEDA status within the first 2 years of second-line treatment as outcome variable.** 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 one year before treatment start showed a trend but was non-significant. (a) From ROC curve analysis (b) Predicted loss of NEDA status. Abbreviations: NAWM = Normal appearing white matter, CI = Confidence interval

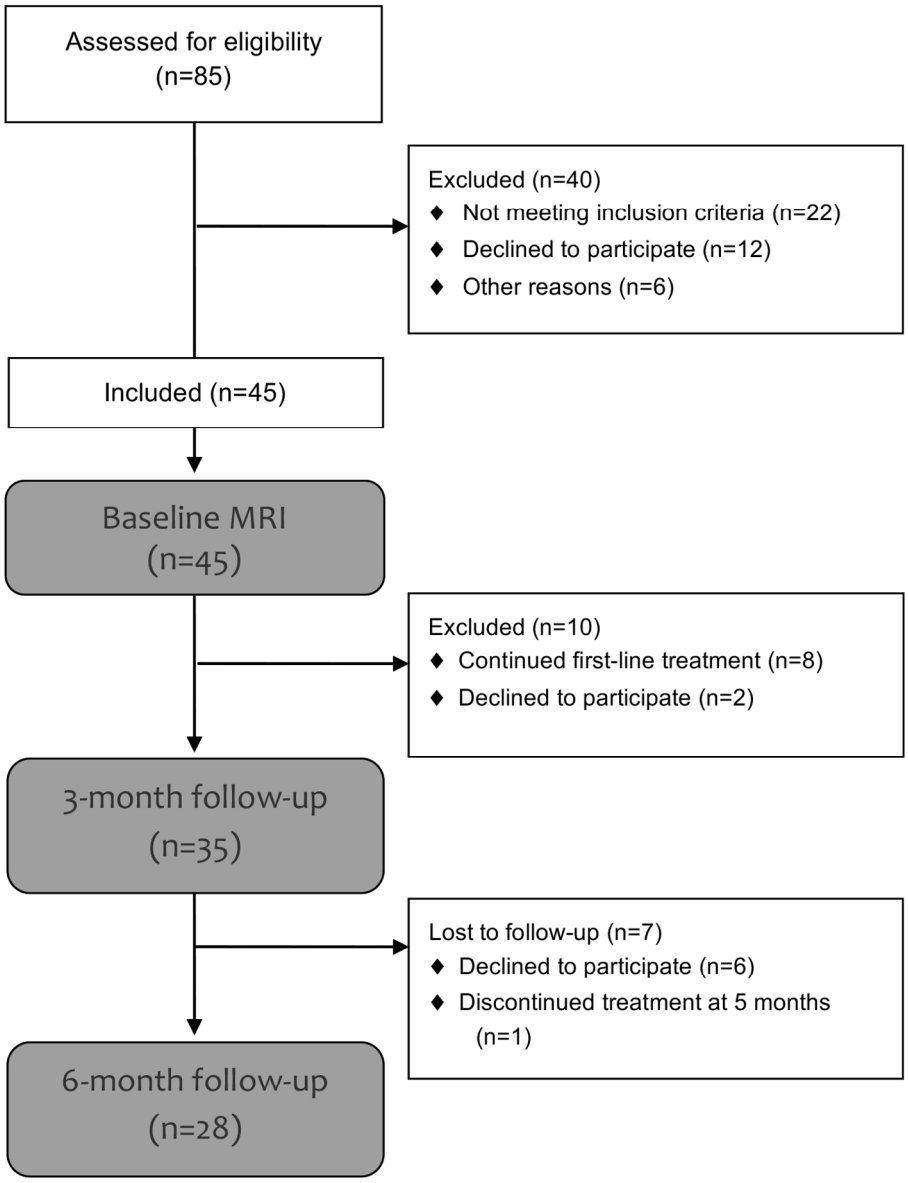

Figure 1 **Subject inclusion procedure.**

125x161mm (300 x 300 DPI)

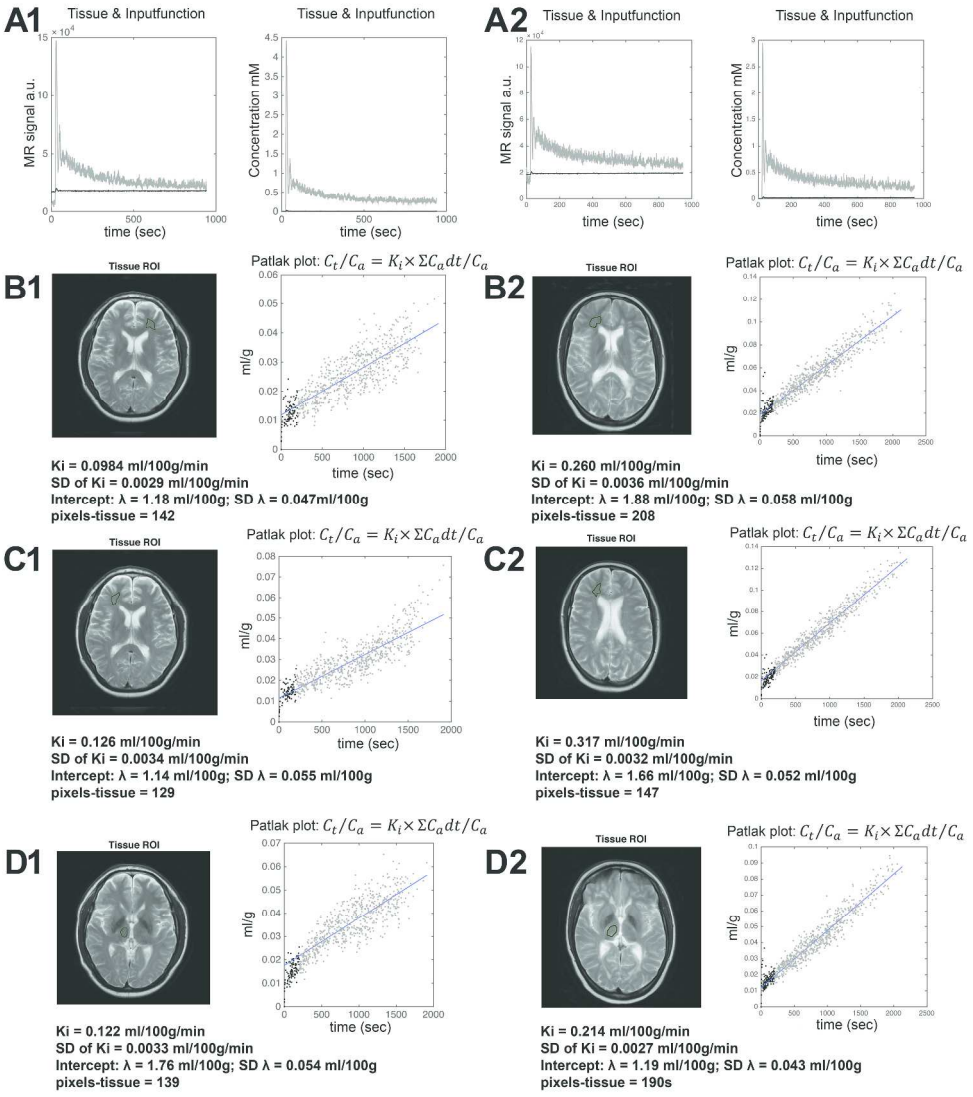

Figure 2 **Arterial input function in arbitrary signal units (A - left) and concentration (A - right), and region of interest placement.** Examples from two subjects, left column from a subject with maintained NEDA status after two years of treatment (A1, B1, C1, and D1) and right column from a subjects with loss of NEDA status after two years of treatment (A2, B2, C2, and D2). Region of interest placement represents two examples in normal appearing white matter (B and C) and one in thalamus (D) with their corresponding Patlak plots at six months post-treatment.

504x570mm (300 x 300 DPI)

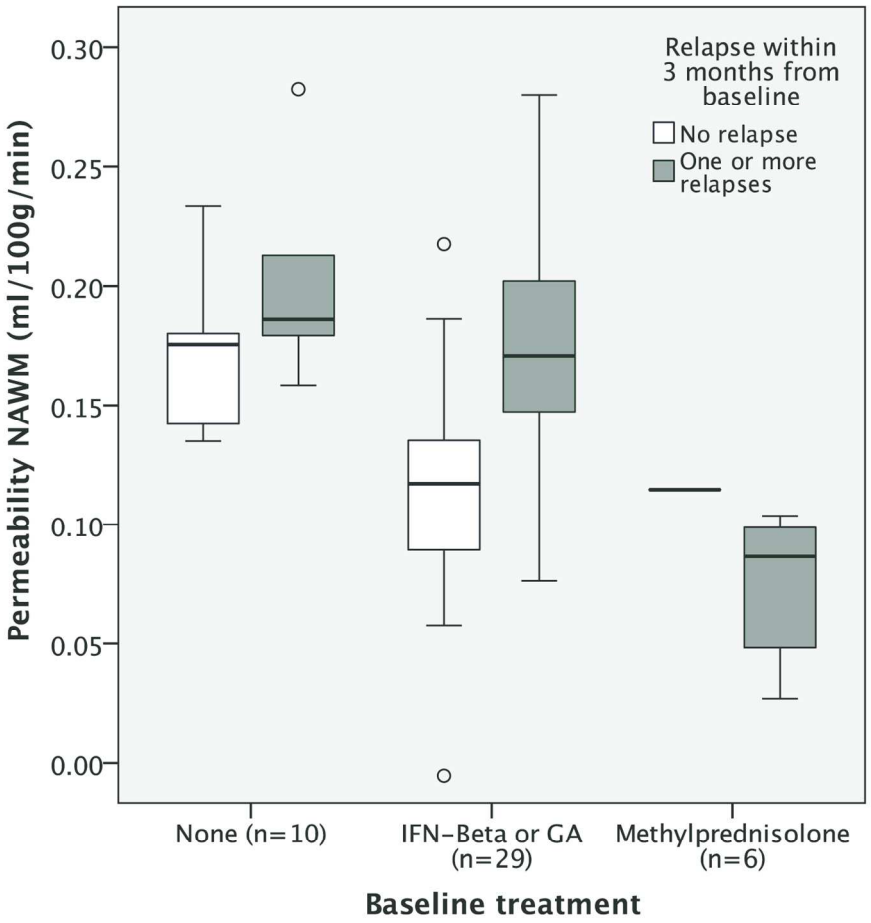

Figure 3 **Baseline permeability in normal appearing white matter (NAWM) for all subjects with a baseline scan (n=45) according to current treatment and recent relapse.** SPSS 23 standard setup for boxplot presentation was used (black line represents the median, boxes represent the interquartile range (IQR: data between the 25 and 75% quartiles). Whiskers represent 1.5 times the IQR. Outliers (open circles) are defined as data points outside 1.5 x IQR). One subject was treated with pulsed steroids (50 mg every first three days/month), and thus received methylprednisolone treatment in spite of not having had a recent relapse. Abbreviations: IFN-Beta = Interferon Beta. GA = Glatiramer acetate.

140x131mm (300 x 300 DPI)

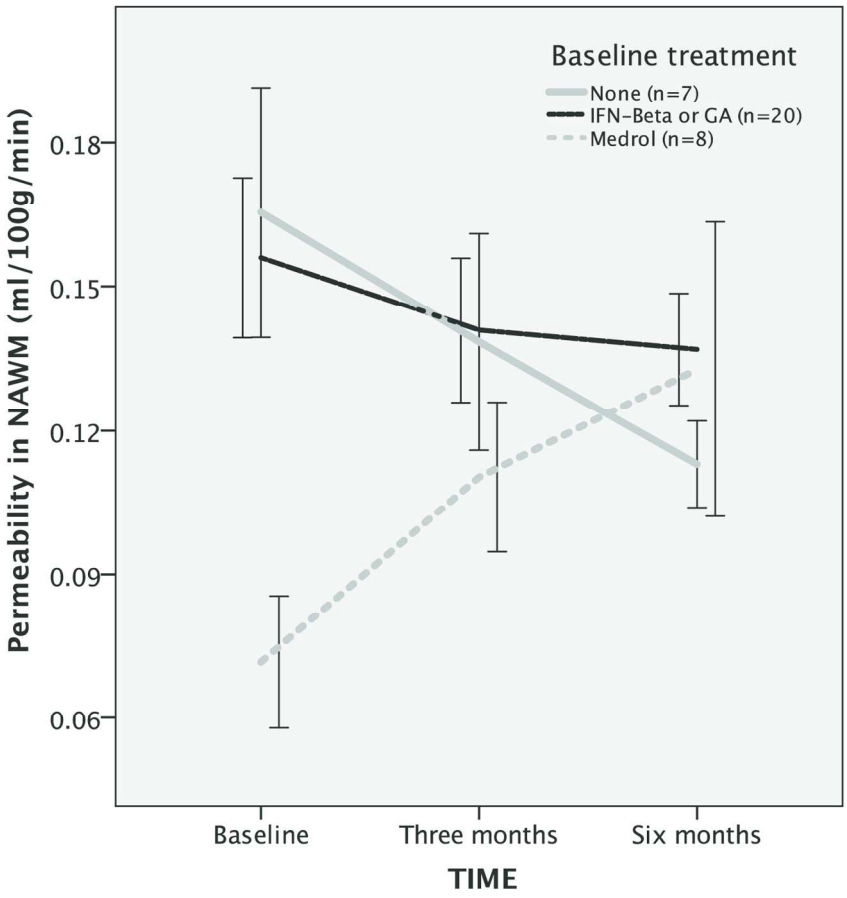

Figure 4 **K_i in periventricular normal-appearing white matter (NAWM) during the course of second line treatment for subjects who completed all three visits (n=27).** Coloured bold lines represent mean Ki according to which treatment the subjects received prior to second-line treatment (grey solid line=no prior treatment, black solid=interferon beta (IFN-Beta) or glatiramer acetate (GA), grey dotted=methylprednisolone within the last two months). Baseline scan was conducted shortly prior to second-line treatment initiation and follow-up scans were conducted at three and six months after second-line treatment. Error bars represent +/- 1 SEM.

143x131mm (300 x 300 DPI)

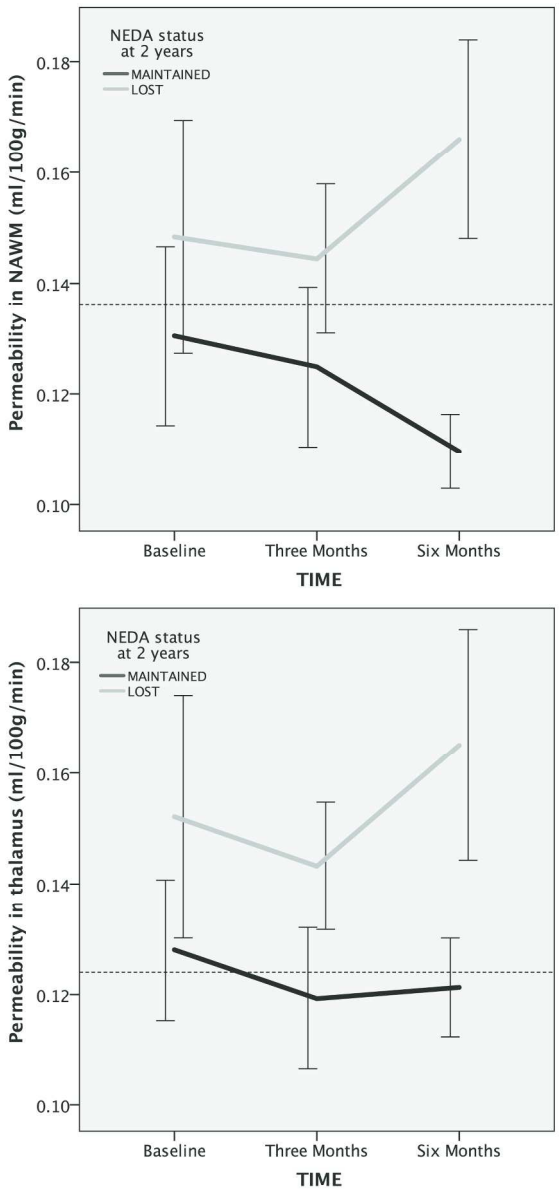

Figure 5 **K_i in periventricular normal-appearing white matter (NAWM; top row) and thalamus (bottom row) pre- natalizumab or fingolimod treatment (baseline) and three and six months post-treatment.** Horizontal dotted line represents optimal threshold for loss of NEDA status from the ROC curve analysis. Black line represents mean Ki in subjects with maintained no-evidence of disease-activity (NEDA) status at two years and grey line represents mean K_i in subjects with lost NEDA status. Error bars represent +/- 1 SEM.

144x263mm (300 x 300 DPI)

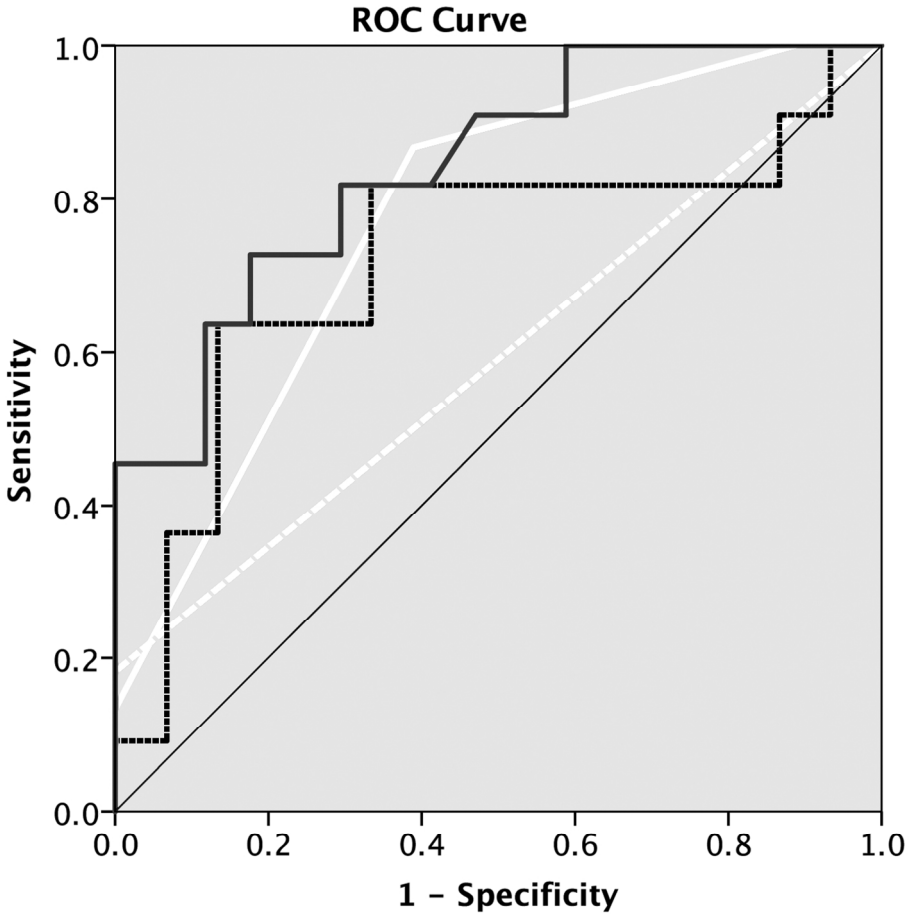

Figure 6 **Result of receiver operator characteristic (ROC) curve analysis with loss of no evidence of disease activity (NEDA) status as outcome variable.** Black solid line= K_i in NAWM at 6 months; Black dotted line= K_i in thalamus at 6 months; White solid line= Annual relapse rate one-year prior to treatment start; White dotted line= New active T2 lesions at 6 months. Abbreviations: AUC = Area under the curve.

130x125mm (300 x 300 DPI)

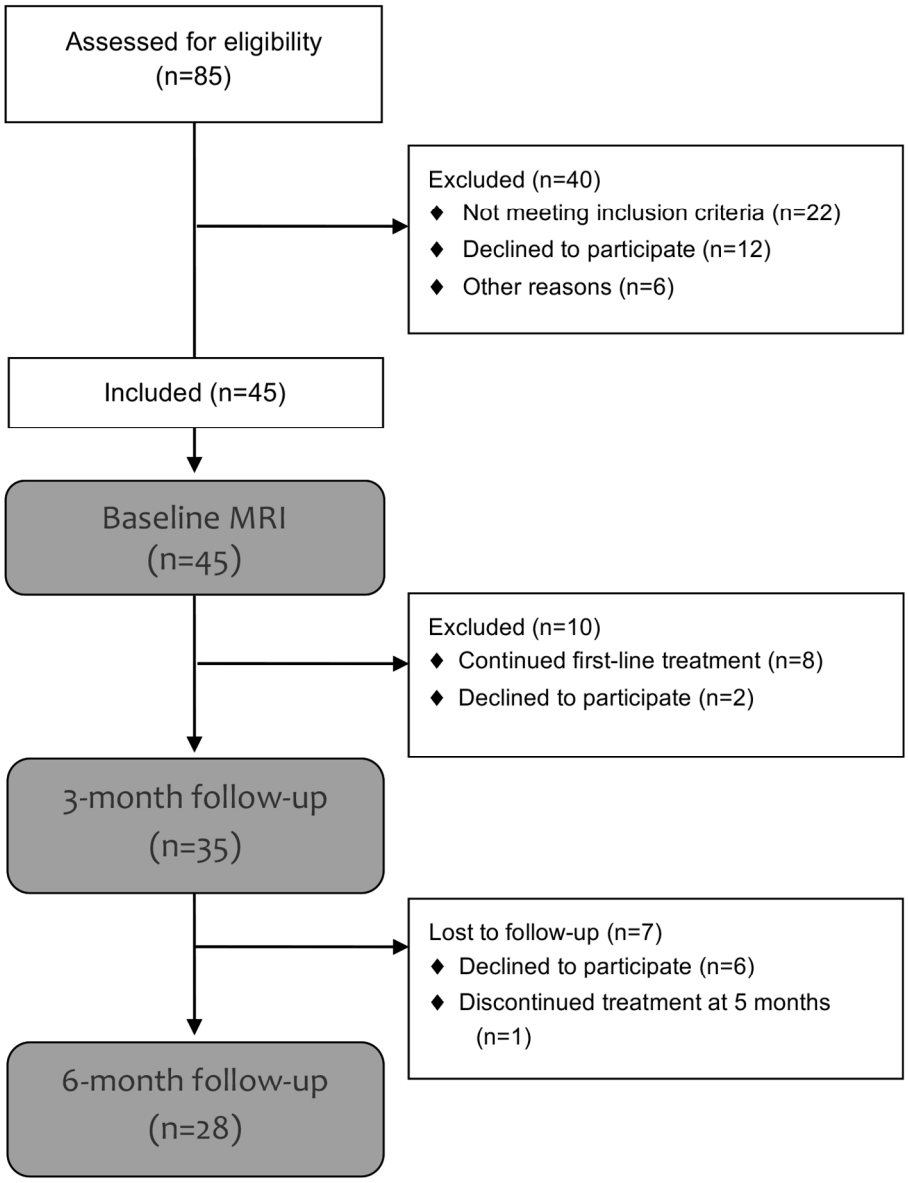

Figure 1 **Subject inclusion procedure.**

125x161mm (300 x 300 DPI)

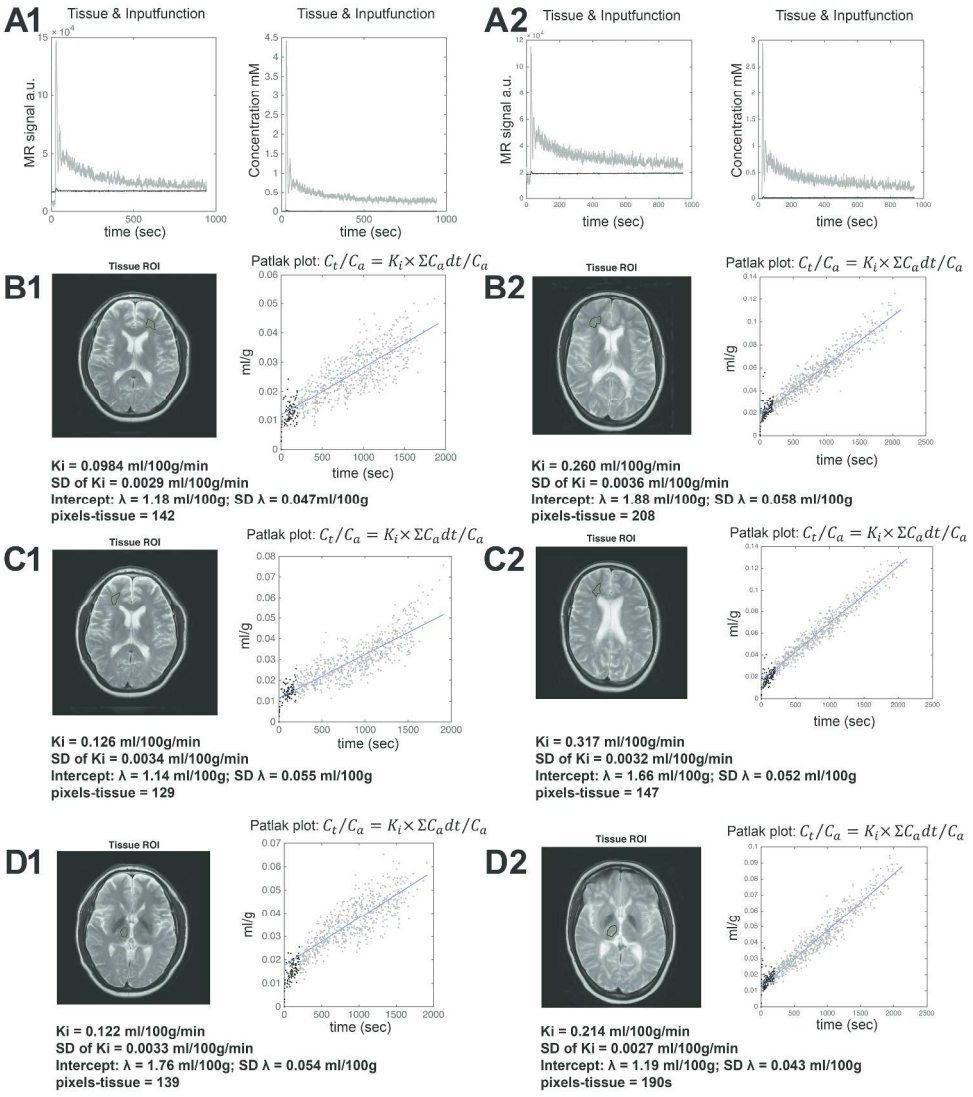

Figure 2 **Arterial input function in arbitrary signal units (A - left) and concentration (A - right), and region of interest placement.** Examples from two subjects, left column from a subject with maintained NEDA status after two years of treatment (A1, B1, C1, and D1) and right column from a subjects with loss of NEDA status after two years of treatment (A2, B2, C2, and D2). Region of interest placement represents two examples in normal appearing white matter (B and C) and one in thalamus (D) with their corresponding Patlak plots at six months post-treatment.

504x570mm (300 x 300 DPI)

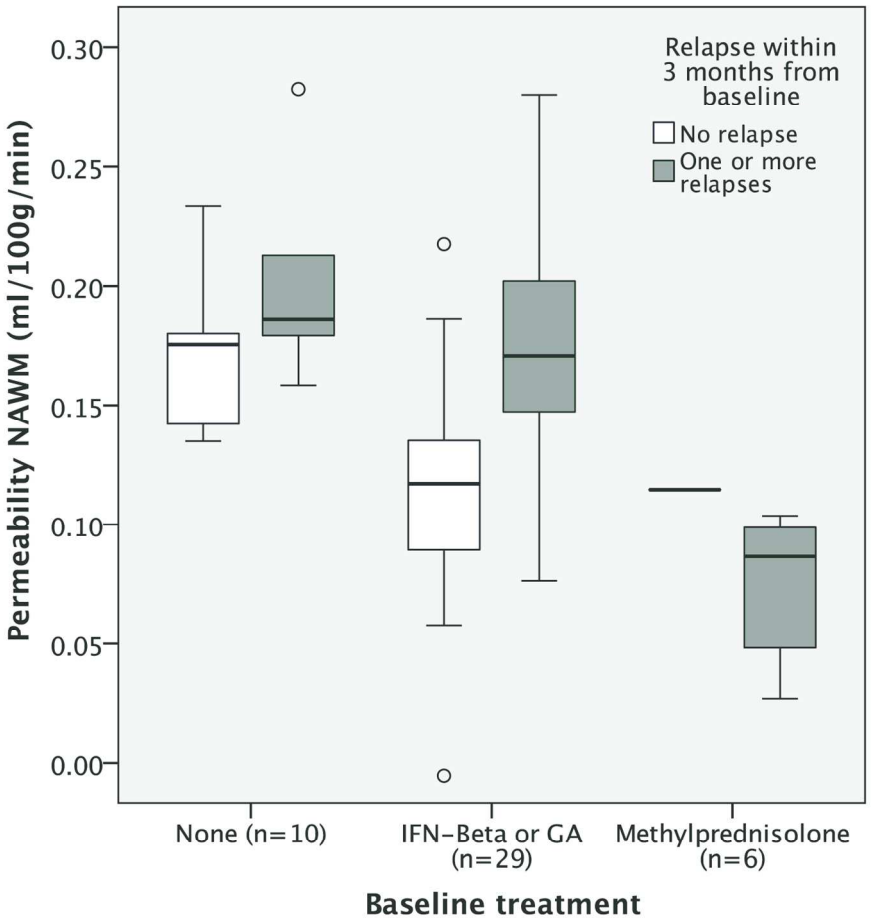

Figure 3 **Baseline permeability in normal appearing white matter (NAWM) for all subjects with a baseline scan (n=45) according to current treatment and recent relapse.** SPSS 23 standard setup for boxplot presentation was used (black line represents the median, boxes represent the interquartile range (IQR: data between the 25 and 75% quartiles). Whiskers represent 1.5 times the IQR. Outliers (open circles) are defined as data points outside 1.5 x IQR). One subject was treated with pulsed steroids (50 mg every first three days/month), and thus received methylprednisolone treatment in spite of not having had a recent relapse. Abbreviations: IFN-Beta = Interferon Beta. GA = Glatiramer acetate.

140x131mm (300 x 300 DPI)

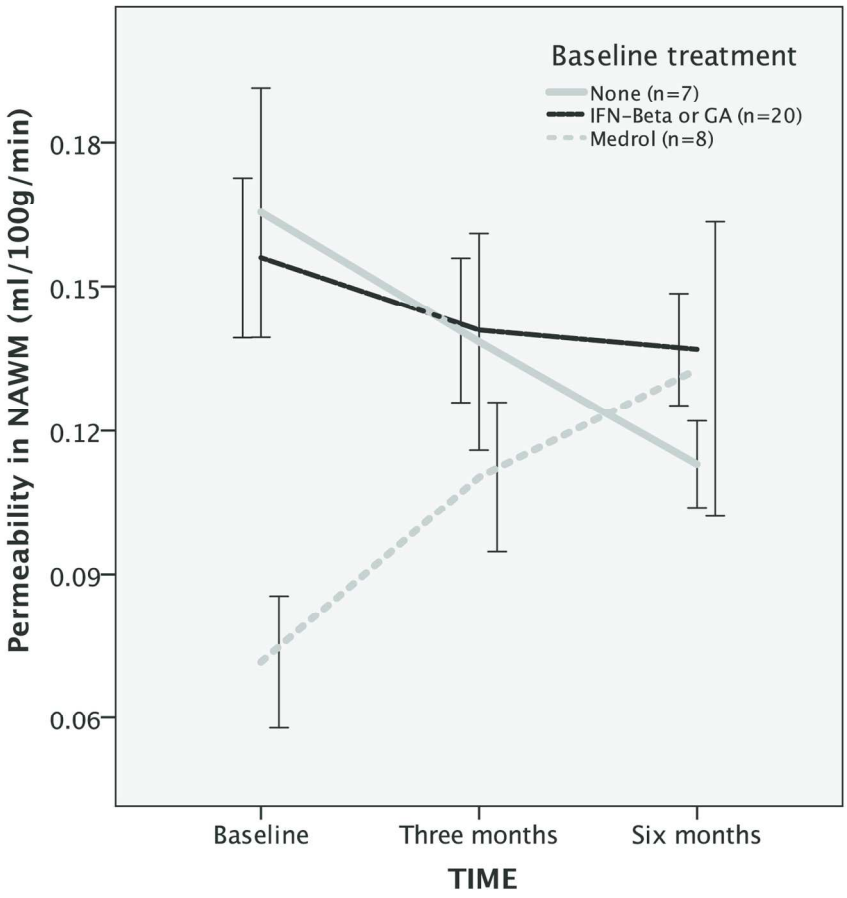

Figure 4 **K_i in periventricular normal-appearing white matter (NAWM) during the course of second line treatment for subjects who completed all three visits (n=27).** Coloured bold lines represent mean Ki according to which treatment the subjects received prior to second-line treatment (grey solid line=no prior treatment, black solid=interferon beta (IFN-Beta) or glatiramer acetate (GA), grey dotted=methylprednisolone within the last two months). Baseline scan was conducted shortly prior to second-line treatment initiation and follow-up scans were conducted at three and six months after second-line treatment. Error bars represent +/- 1 SEM.

143x131mm (300 x 300 DPI)

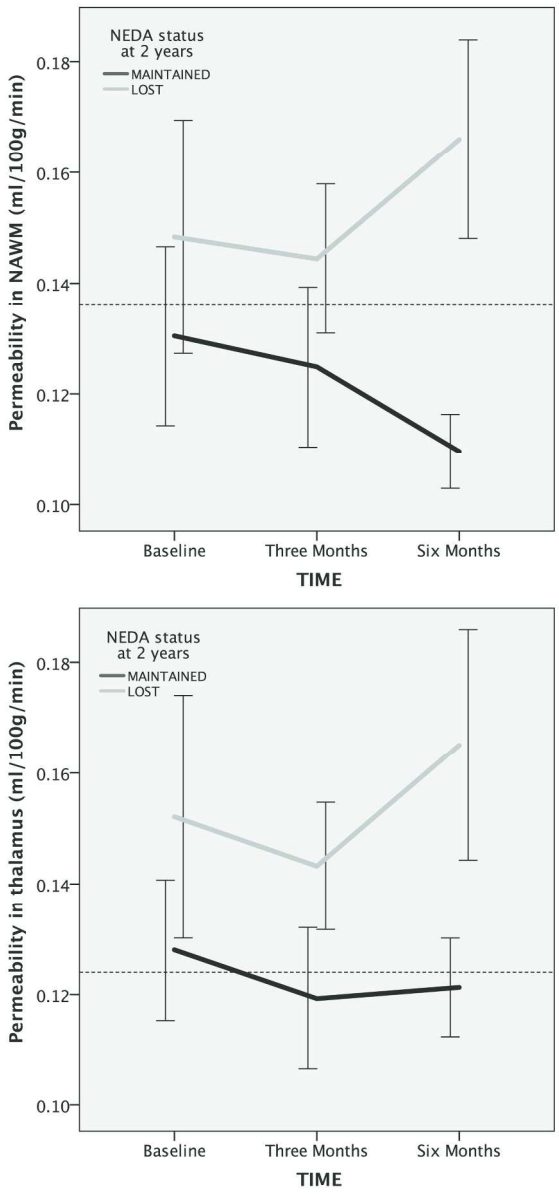

Figure 5 **K_i in periventricular normal-appearing white matter (NAWM; top row) and thalamus (bottom row) pre- natalizumab or fingolimod treatment (baseline) and three and six months post-treatment.** Horizontal dotted line represents optimal threshold for loss of NEDA status from the ROC curve analysis. Black line represents mean Ki in subjects with maintained no-evidence of disease-activity (NEDA) status at two years and grey line represents mean K_i in subjects with lost NEDA status. Error bars represent +/- 1 SEM.

144x263mm (300 x 300 DPI)

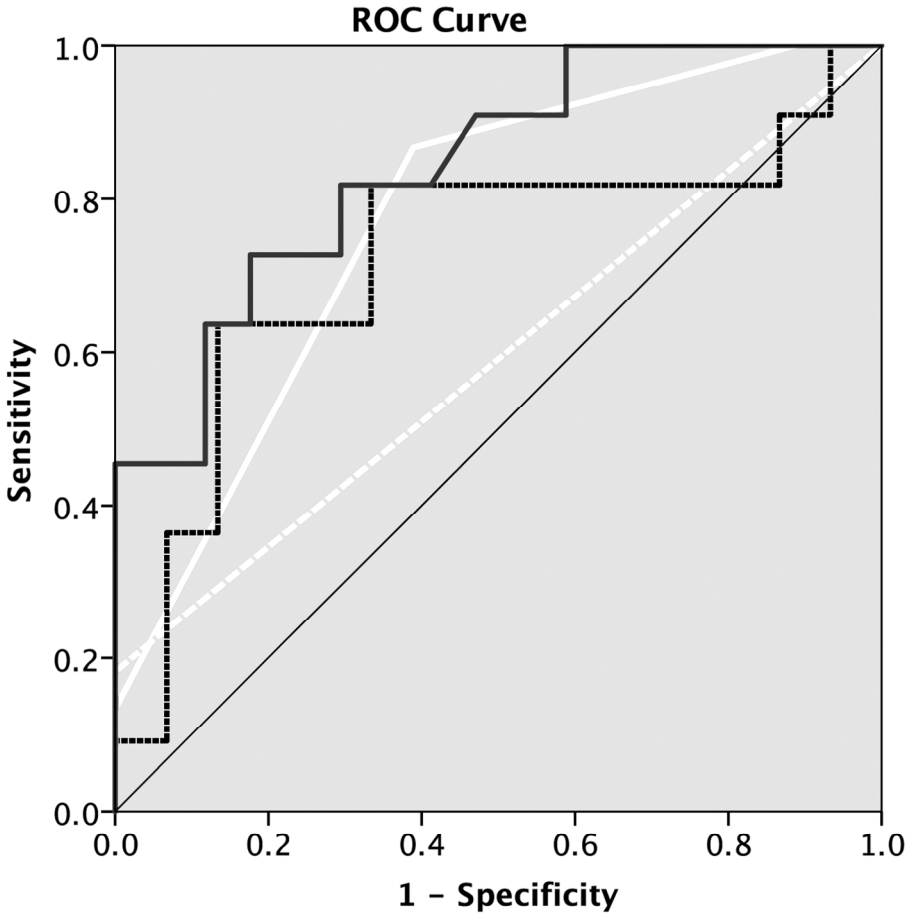

Figure 6 **Result of receiver operator characteristic (ROC) curve analysis with loss of no evidence of disease activity (NEDA) status as outcome variable.** Black solid line= K_i in NAWM at 6 months; Black dotted line= K_i in thalamus at 6 months; White solid line= Annual relapse rate one-year prior to treatment start; White dotted line= New active T2 lesions at 6 months. Abbreviations: AUC = Area under the curve.

130x125mm (300 x 300 DPI)

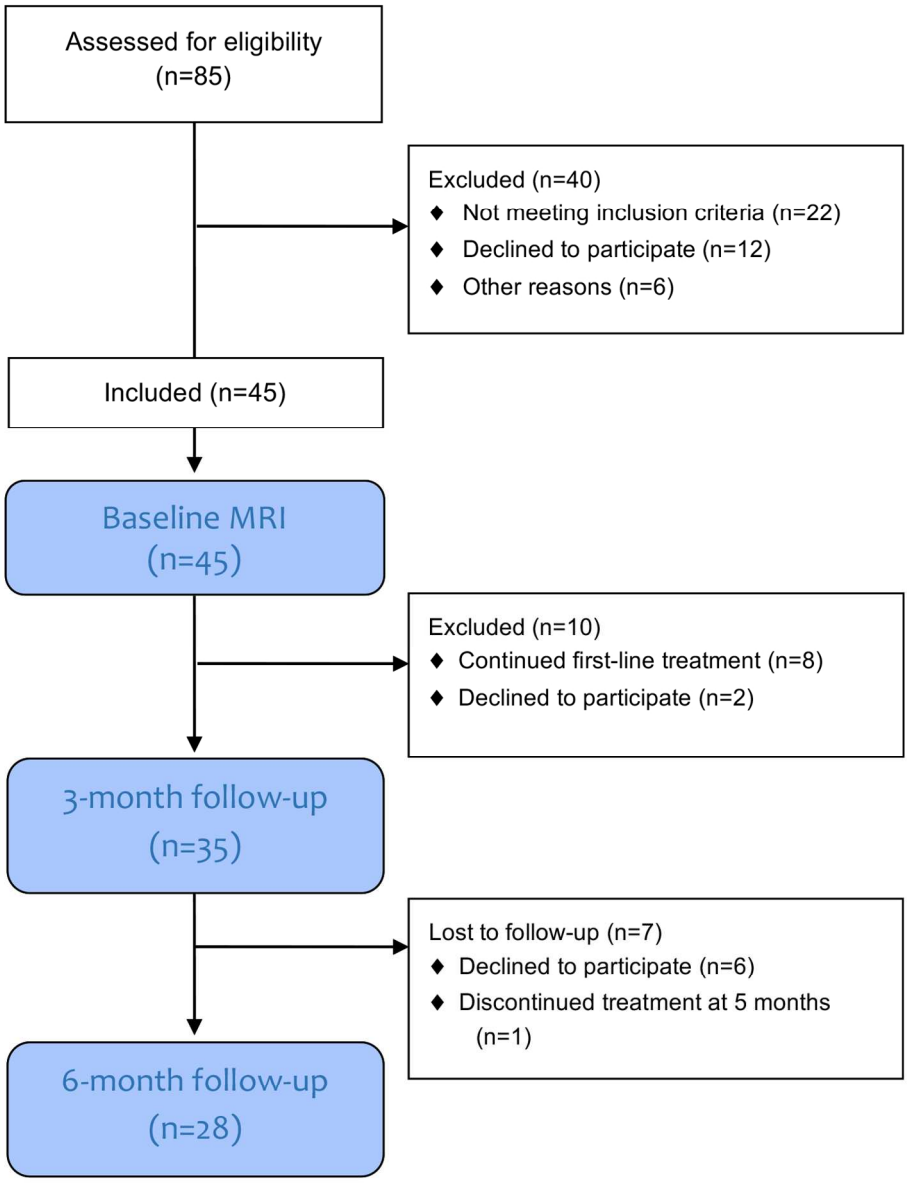

Figure 1 **Subject inclusion procedure.**

125x161mm (300 x 300 DPI)

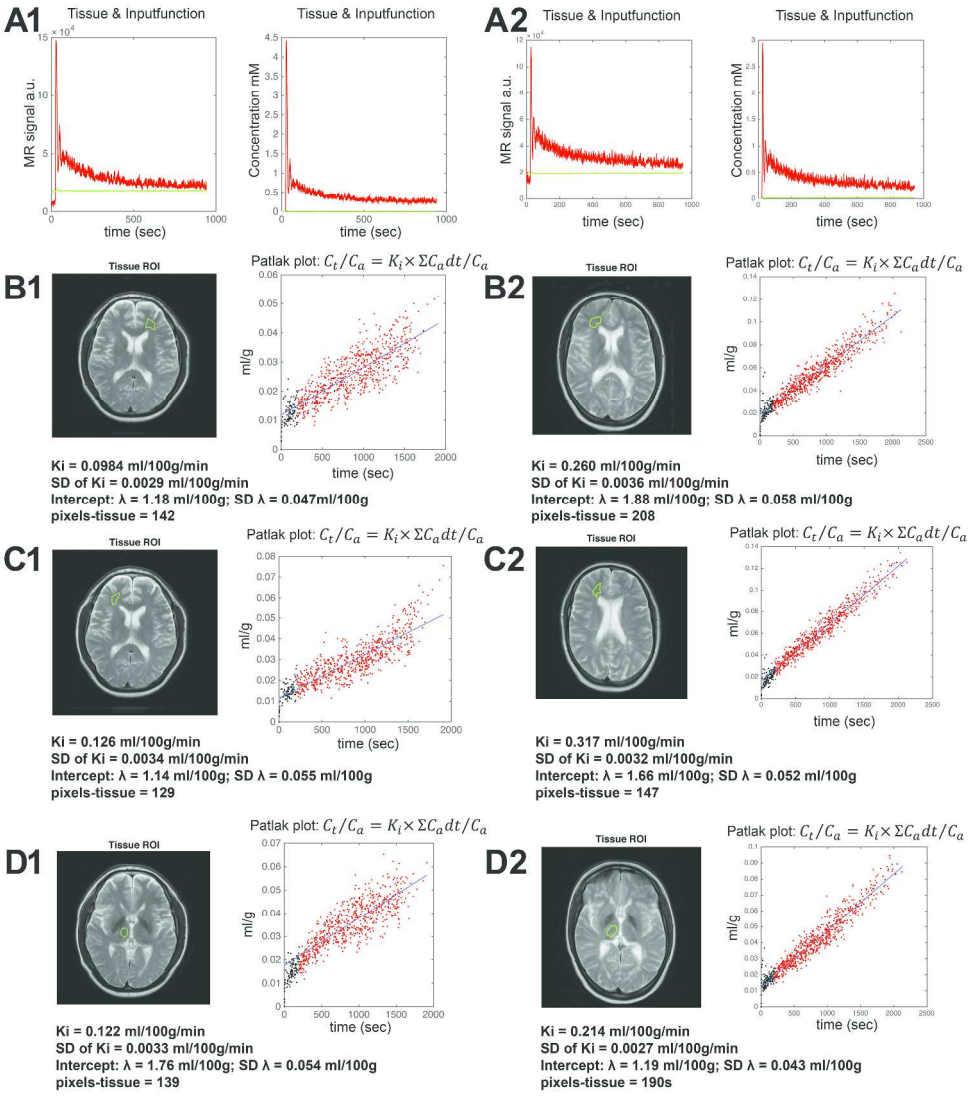

Figure 2 **Arterial input function in arbitrary signal units (A - left) and concentration (A - right), and region of interest placement.** Examples from two subjects, left column from a subject with maintained NEDA status after two years of treatment (A1, B1, C1, and D1) and right column from a subjects with loss of NEDA status after two years of treatment (A2, B2, C2, and D2). Region of interest placement represents two examples in normal appearing white matter (B and C) and one in thalamus (D) with their corresponding Patlak plots at six months post-treatment.

504x570mm (300 x 300 DPI)

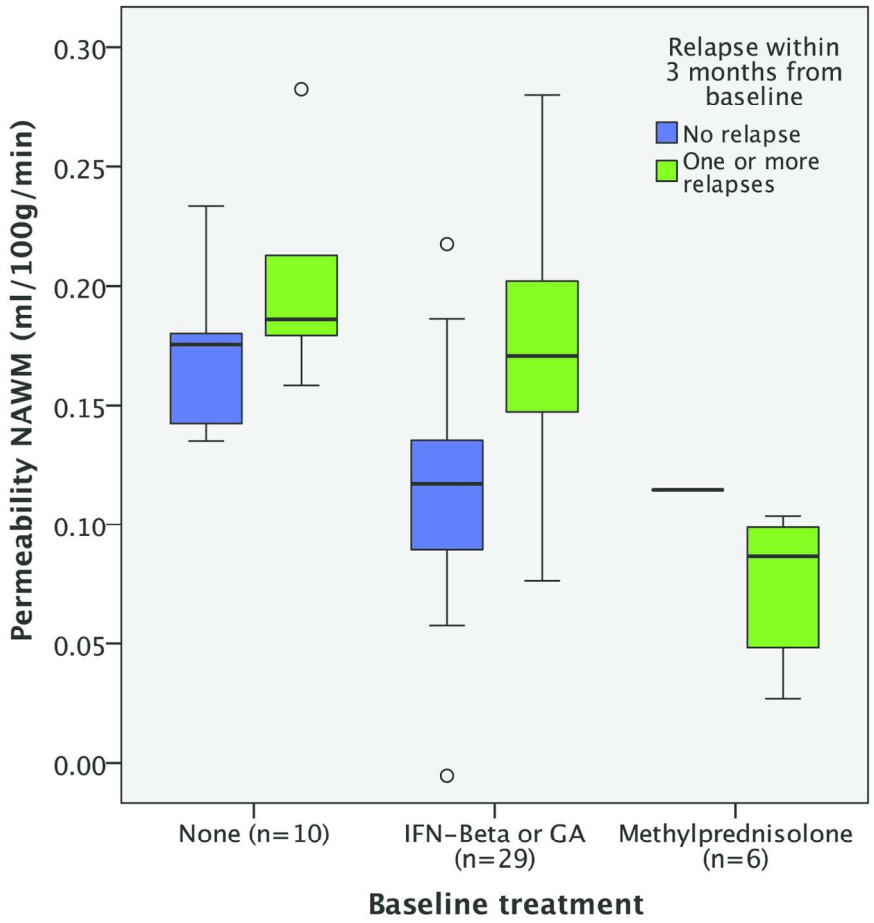

Figure 3 **Baseline permeability in normal appearing white matter (NAWM) for all subjects with a baseline scan (n=45) according to current treatment and recent relapse.** SPSS 23 standard setup for boxplot presentation was used (black line represents the median, boxes represent the interquartile range (IQR: data between the 25 and 75% quartiles). Whiskers represent 1.5 times the IQR. Outliers (open circles) are defined as data points outside 1.5 x IQR). One subject was treated with pulsed steroids (50 mg every first three days/month), and thus received methylprednisolone treatment in spite of not having had a recent relapse. Abbreviations: IFN-Beta = Interferon Beta. GA = Glatiramer acetate.

140x131mm (300 x 300 DPI)

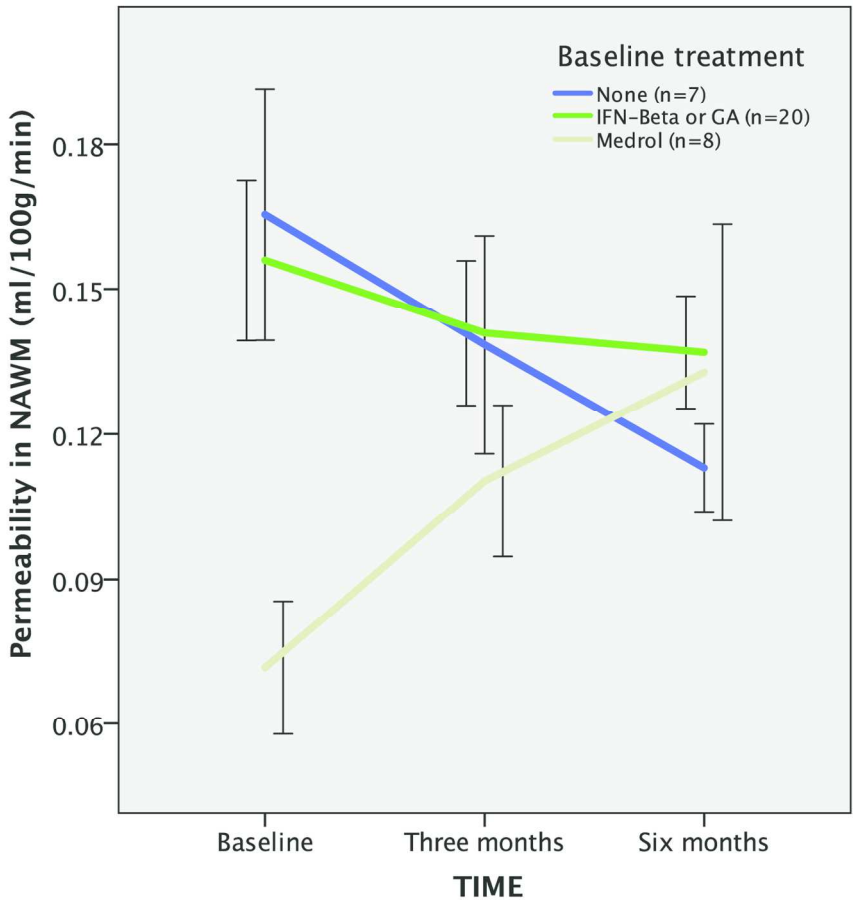

Figure 4 **K_i in periventricular normal-appearing white matter (NAWM) during the course of second line treatment for subjects who completed all three visits (n=27).** Coloured bold lines represent mean Ki according to which treatment the subjects received prior to second-line treatment (blue=no prior treatment, green=interferon beta (IFN-Beta) or glatiramer acetate (GA), brown=methylprednisolone within the last two months). Baseline scan was conducted shortly prior to second-line treatment initiation and follow-up scans were conducted at three and six months after second-line treatment. Error bars represent +/- 1 SEM.

142x131mm (300 x 300 DPI)

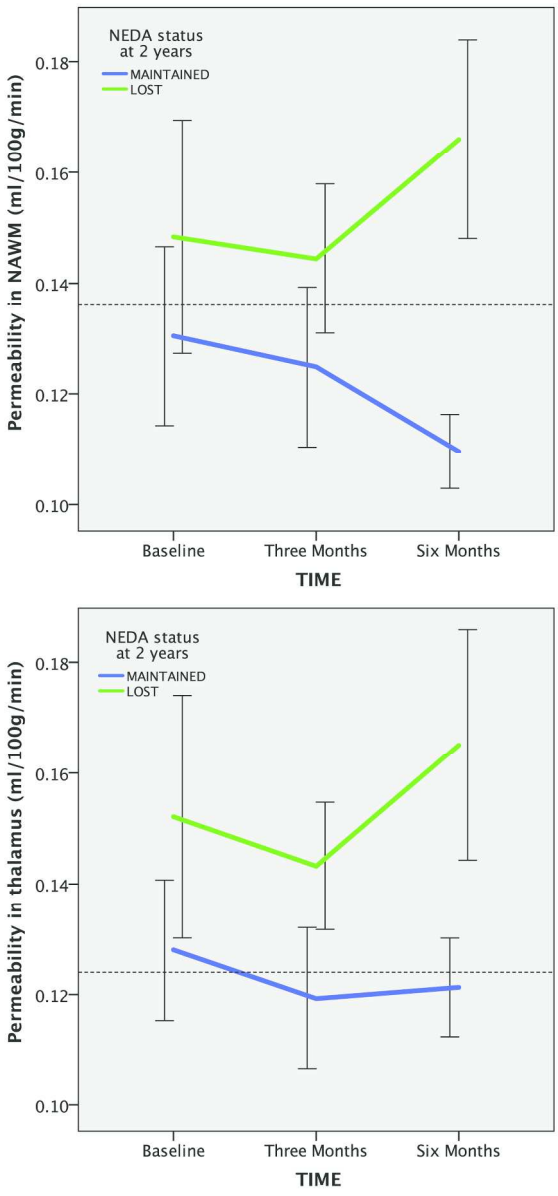

Figure 5 **K_i in periventricular normal-appearing white matter (NAWM; top row) and thalamus (bottom row) pre- natalizumab or fingolimod treatment (baseline) and three and six months post-treatment.** Horizontal dotted line represents optimal threshold for loss of NEDA status from the ROC curve analysis. Blue line represents mean Ki in subjects with maintained no-evidence of disease-activity (NEDA) status at two years and green line represents mean K_i in subjects with lost NEDA status. Error bars represent +/- 1 SEM.

141x263mm (300 x 300 DPI)

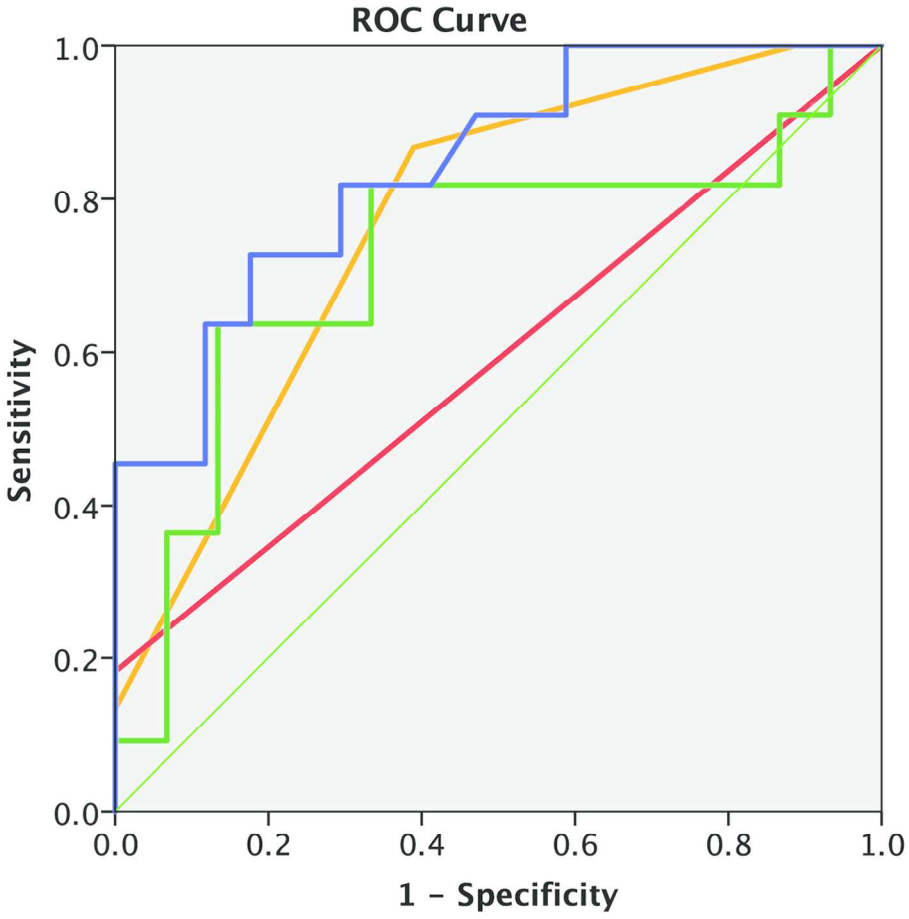

Figure 6 **Result of receiver operator characteristic (ROC) curve analysis with loss of no evidence of disease activity (NEDA) status as outcome variable.** Blue= K_i in NAWM at 6 months; Green = K_i in thalamus at 6 months; Orange= Annual relapse rate one-year prior to treatment start; Red= New active T2 lesions at 6 months. Abbreviations: AUC = Area under the curve.

130x125mm (300 x 300 DPI)