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The clinical response to minocycline in multiple sclerosis is accompanied by beneficial immune changes: a pilot study

RK Zabad¹, LM Metz^{1†}, TR Todoruk¹, Y Zhang², JR Mitchell², M Yeung¹, DG Patry¹, RB Bell¹ and VW Yong^{1†}

Minocycline has immunomodulatory and neuroprotective activities *in vitro* and in an animal model of multiple sclerosis (MS). We have previously reported that minocycline decreased gadolinium-enhancing activity over six months in a small trial of patients with active relapsing–remitting MS (RRMS). Here we report the impact of oral minocycline on clinical and magnetic resonance imaging (MRI) outcomes and serum immune molecules in this cohort over 24 months of open-label minocycline treatment. Despite a moderately high pretreatment annualized relapse rate (1.3/year pre-enrolment; 1.2/year during a three-month baseline period) prior to treatment, no relapses occurred between months 6 and 24. Also, despite very active MRI activity pretreatment (19/40 scans had gadolinium-enhancing activity during a three-month run-in), the only patient with gadolinium-enhancing lesions on MRI at 12 and 24 months was on half-dose minocycline. Levels of the p40 subunit of interleukin (IL)-12, which at high levels might antagonize the proinflammatory IL-12 receptor, were elevated over 18 months of treatment, as were levels of soluble vascular cell adhesion molecule-1. The activity of matrix metalloproteinase-9 was decreased by treatment. Thus, clinical and MRI outcomes are supported by systemic immunological changes and call for further investigation of minocycline in MS. *Multiple Sclerosis* 2007; 13: 517–526. <http://msj.sagepub.com>

Key words: adhesion molecule; interleukin-12; MMP; multiple sclerosis; neuroimmunology; therapy

Introduction

Tetracyclines are antibiotics that are commonly used to treat chronic conditions such as acne. Second-generation tetracyclines, such as doxycycline and minocycline, have better pharmacokinetic properties than their parent, tetracycline, when used orally. Indeed, minocycline is lipid-soluble and crosses the blood–brain barrier well [1,2]. In recent years, minocycline has been shown to have remarkable neuroprotective activity whereby it alleviates the neurodegeneration occurring in animal models of stroke, Huntington's disease, amyotrophic lateral sclerosis, Parkinson's disease and spinal cord injury [3]. In an animal

model of MS, experimental autoimmune encephalomyelitis (EAE), we and others found that minocycline attenuates clinical severity of disease and reduces several neuropathologic outcomes [4–6].

The efficacy of minocycline in a wide spectrum of animal models of neurological diseases is attributed to several properties, including the inhibition of microglia activation and the decreased activity of several enzyme systems such as inducible nitric oxide synthase (iNOS) and matrix metalloproteinases (MMPs) [3]. Minocycline also reduces activity of T lymphocytes and monocytes [7]. Because minocycline is effective in animal models of induced disease where the pathogen is not an infectious agent, it is unlikely that efficacy is due to

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its antibiotic activity, although this cannot be excluded as a contributing factor.

Because of its immunomodulatory and neuroprotective activities, and its effectiveness in EAE, we tested minocycline in 10 patients with active relapsing–remitting multiple sclerosis (RRMS). During a pre-planned treatment extension to 36 months, serum was collected at –3, 0, 3, 6, 12 and 18 months to evaluate for several markers of the biological impact of treatment. In this manuscript we report the results of this biomarker evaluation along with the corresponding clinical and gadolinium-enhancing magnetic resonance imaging (MRI) outcomes over two years. Immune data at the two-year mark were not collected. Our results confirm that minocycline affects several immunological parameters in RRMS and continues to look promising as a safe therapy to potentially reduce MRI gadolinium-enhancing lesions and clinical relapse frequency. These studies also identify additional mechanisms by which minocycline may act in MS.

Materials and methods

Patients

This was an open-label, single-centre study, in which ten subjects with RRMS were treated with oral minocycline 100 mg twice daily for six months following a three-month run-in period. The maximum approved dose of minocycline was chosen for study as it is known to be safe for chronic use in other conditions. Inclusion criteria were age between 18 and 50 years, a history of at least two relapses within the prior 24 months and an Extended Disability Status Scale (EDSS) score between 0 and 5.5. Exclusion criteria were treatment with corticosteroids during the prior month, the presence of any unstable medical or psychiatric disorder, use of interferon- β (IFN- β), glatiramer acetate or IV gamma globulin within six months prior to enrolment, use of any investigational drug or other immunosuppressive therapy within 12 months prior to baseline, any prior use of cladribine or total lymphoid irradiation, allergy to tetracyclines or gadolinium, pregnancy or breastfeeding. All patients declined approved disease-modifying therapy and provided signed informed consent. The study was approved by the Calgary research ethics board.

Study design

EDSS and clinical and laboratory assessments were completed at three-month intervals (enrolment,

month 0, month 3, month 6) initially, then at six-month intervals to 36 months. An unblinded treating neurologist was responsible for the overall medical management of each patient and for EDSS scoring. Clinical evaluation included vital signs, physical examination, haematology and biochemistry for safety. Serum was collected at months –3, 0, 3, 6, 12 and 18 and frozen at –70°C. MRI was performed at enrolment, at four-week intervals during the 3 month run-in and 6-month treatment periods, then at 12, 24 and 36 months. Patients without MRI activity during the run-in phase continued in the study to confirm lack of MRI worsening during treatment. Three neurologists uninvolved with patient management were available to review serious adverse events and MRI worsening.

All MR images were obtained from a 3.0-T MR scanner (Signa; GE Medical Systems, Waukesha, WI). Each MR scan included: oblique axial dual echo spin-echo (TR/TE1/TE2: 2716/20/80 ms) proton density weighted (PDw) and T2-weighted (T2w) images and axial spin-echo T1-weighted images (TR/TE: 650/8 ms) before, and 5 min after IV injection of 0.1 mmol/kg GD-DTPA. Common scan parameters were: acquisition matrix, 512 \times 192; reconstructed matrix, 512 \times 512; field of view, 24 cm; voxel dimensions, 0.46 mm in-plane; and slice thickness, 3 mm without gap. This yielded 50 contiguous images for each sequence. Analysis was performed by a single neuroradiologist using a semi-automatic computer-assisted lesion quantification program (Segtool[®]) [9], which reduces the variability of MR lesion identification and volume measurement [10]. Analysis was not blinded, as the study was known to be open-label.

The primary outcome was change in the mean number of gadolinium-enhancing lesions during the first six months of treatment compared to the run-in period. A relapse was defined as the appearance of a new symptom, or worsening of an old symptom, attributable to MS, accompanied by objective neurological dysfunction, lasting at least 24 h, in the absence of fever. The event had to be preceded by stability or improvement for at least 30 days.

Determination of serum biomarkers

Interleukin (IL)-12 is made up of a heterodimer of p35 and p40 subunits and the mature cytokine is referred to as IL-12p70. Commercially available ELISA kits were used to determine the concentration of IL-12p40 or IL-12p70 (Biosource International). The IL-12p40 ELISA recognizes the IL-12p70 heterodimer, IL-12p40 subunit and the IL-12 (p40)₂ homodimer. Levels of tumour necrosis factor

(TNF)- α , IFN- γ and IL-10 in sera were determined using ELISA (Biosource International).

High-sensitivity ELISA kits were utilized to measure soluble vascular cell adhesion molecule (sVCAM)-1 and intercellular cell adhesion molecule 1 (sICAM-1) (Bender Medsystems). Levels of MMP-9 and one of its endogenous antagonists, tissue inhibitor of metalloproteinases (TIMP)-1, were assayed using ELISA (R&D systems).

All ELISA determinations were conducted according to the manufacturer's instructions. All samples from an individual patient were analysed on a single plate to minimize the possibility of interplate variability. These samples were not coded and were performed in duplicates. Fifty and 100 μ L of serum were used for IL-12p40 and IL-12p70 assays, respectively. Serum diluted 1/100 was used for sICAM-1, MMP-9 and TIMP-1 and serum diluted 1/50 was used for sVCAM-1. We did not encounter high non-specific binding in ELISA assays using undiluted sera, when saline samples were compared as controls. Thus, no additional steps were necessary to reduce background. We found also good intra- and interplate consistency, and we used standard curves on all ELISA plates to ensure that measurements were obtained below curve saturation.

MMP-9 activity assay

While ELISA provides information on MMP-9 content, it does not reveal the actual enzymatic activity of MMP-9. Thus, we utilized an assay based on the capacity of MMP-9 to degrade a fluorogenic substrate, DNP-Pro-Cha-Gly-Cys(Me)-His-Ala-Lys(N-Me-Abz)-NH₂ (Cha, *b*-cyclohexylalanyl; Abz, 2-aminobenzoyl (anthraniloyl); DNP, 2,4-dinitrophenyl) (Calbiochem). In its intact form, the substrate has a fluorophore (Abz) and a quencher (DNP) bound through a sequence of amino acids recognized and cleaved by MMP-9. Upon cleavage by MMP-9 at the Cys (Me)-His bond, the quencher (DNP) is removed from the fluorophore (Abz) to produce an evolving fluorescent signal. Serum proteolytic activity for this substrate can be specifically blocked by an MMP-9 inhibitor, indicating that the fluorescent signal is of MMP-9 activity in serum (data not shown). The basis of this assay has been previously described by others [11].

In the current study, undiluted serum (98 μ L) was combined with 6.5 μ L MMP fluorogenic substrate (in DMSO, 130 μ M) in a black 96-well plate. All samples from individual patients were run on the same plate. Recombinant active MMP-9 (83 kDa, >95% activated) (Oncogene) diluted with saline (stock concentration of 290 ng/mL) was used to standardize between plates. Five microlitres of the stock MMP-9 solution was combined

with saline (93 μ L) and fluorogenic substrate (6.5 μ L) and used as a positive control on each plate. All sera values were normalized to this control by dividing serum activity by the measured activity of MMP-9 multiplied by 100. This standardization corrects for equipment variation, and allows for comparison of patient data when assays were performed at different times.

The evolving signal produced by MMP-9 cleavage of the fluorogenic substrate was recorded every 2 min for 2 h. The reaction was carried out at 37°C in a Gemini XPS fluorimeter (Molecular Devices Corporation, Sunnyvale, CA), at excitation and emission wavelengths of 330 and 450 nm respectively. The evolving signal produced by serum MMP-9 cleavage of the fluorogenic substrate was analysed using Michaelis-Menten kinetics; V_{max} , the maximum attainable rate of the reaction and K_m , a measure of the enzyme's substrate affinity, are reported.

Statistical analysis

Clinical results will be presented descriptively. For serum studies, the 'pretreatment' group corresponds to samples from -3 and 0 months and the 'on-treatment' group corresponds to sera from 3, 6, 12 and 18 months. Comparison between the pretreatment and on-treatment groups was tested with the Wilcoxon matched-pairs signed-ranks test. Significance for within-group changes was tested with repeated measures non-parametric ANOVA (Friedman's test) and post-hoc with the Wilcoxon matched-pairs signed-ranks test. Alpha was set at 0.05 for the overall group and 0.01 for between time-point comparison, using the Bonferroni correction (α^3), where α is the level of significance for the overall group, and α is the level of significance for between time-comparison groups).

Correlation between MMP-9 activity and IL-12p40 level was calculated using linear regression analysis. The correlation coefficient (r) and 95% confidence intervals were calculated to establish significance.

Results

Clinical and MRI outcomes

Ten patients were enrolled. Eighty percent of participants were women, mean age was 42.8 years (SD 4.0) and mean MS duration was 11.8 years (SD 6.3). Median EDSS at baseline was 2.5 (range 1.5-5.5) and mean relapse rate in the two years prior to enrolment was 2.6 (range 2-3). There were no serious adverse events or laboratory abnormalities

over the 24-month period. Treatment was generally well tolerated, although at onset of dosing five patients experienced transient, mild lightheadedness and nausea, which was managed in two patients by a two-week dose reduction. In one patient (patient 1) persistent nausea necessitated reduction of minocycline dose to 50 mg twice daily between months 6 and 24.

The relapse and MRI outcomes for each patient are illustrated in Figure 1. Patient 5 declined the 12-month scan. Patient 7 did not continue in the trial extension because of inconvenience, as she lives in a different city. Patient 8 discontinued treatment, but not follow-up after month 18 during transient worsening of pre-existing MS symptoms (truncal dysaesthesia) that accompanied a flu shot and which she attributed to the minocycline. Patient 10 declined gadolinium beyond the run-in phase due to venous irritation. Thus, clinical outcomes are available between 6 and 24 months for nine patients and MRI gadolinium-enhancing activity is available for eight patients.

The pretreatment two-year relapse number was relatively high (mean of 2.6; annualized rate 1.3), remained high during the three-month run-in period (3 relapses; annualized rate 1.2), and did not drop during the first six-month treatment period (5 relapses; annualized relapse rate 1.0) [8], but none of these patients experienced a relapse between months 6 and 24. Also, none developed

progressive disease course or had EDSS worsening of one or more point.

Monthly MRI scans showed no enhancing activity between months 3 and 6 [8] and the only MRI scans after month 3 that showed enhancing activity were those of one patient (patient 1) receiving reduced dose minocycline. These two scans each had a single gadolinium-enhancing lesion. This supports MRI data from the initial 6-month treatment period where there was a relative reduction of greater than 84% in the mean total enhancing lesion number when the run-in phase (1.38 lesions per scan) was compared to the treatment phase (0.22 lesions per scan) (Wilcoxon signed-rank test, $z = 2.204$, $P = 0.0276$).

Inflammatory cytokines during minocycline treatment

Minocycline treatment significantly increased the level of IL-12p40 (Table 1) when pretreatment samples were compared to samples obtained during treatment (the treatment group). The increase was also statistically significant at the majority of post-treatment time points (Table 2). Figure 2 demonstrates the profile of IL-12p40 in individual patients by treatment duration; it is noteworthy that levels continue to rise during treatment in most patients. Of interest is patient 1 on half dose minocycline

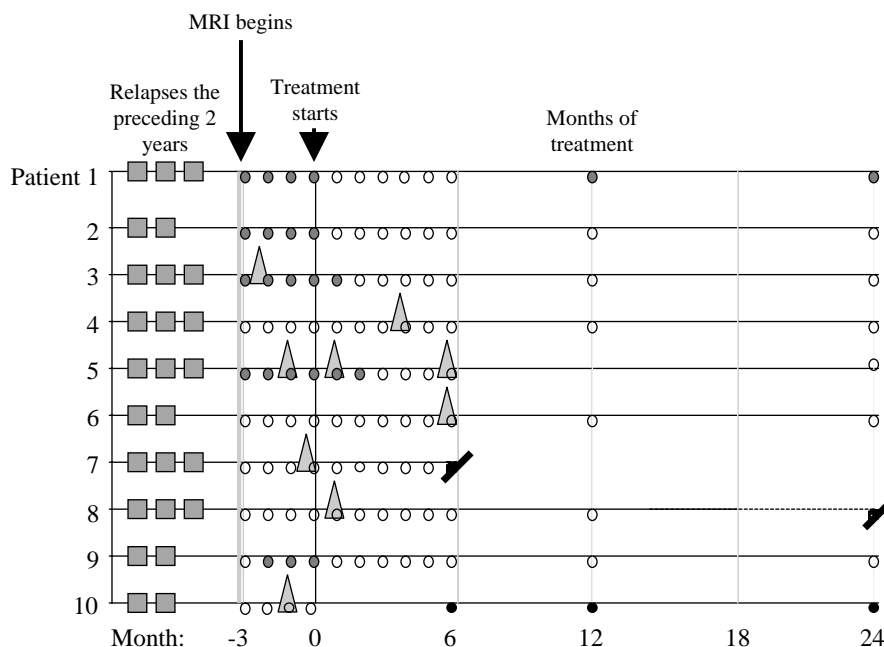


Figure 1 The effect of minocycline on relapses and MRI gadolinium enhancement up to 24 months. The course of each patient is displayed horizontally. ■ indicates a relapse during the two years before enrolment, ▲ indicates a relapse on treatment, ○ indicates an MRI (empty if non-enhancing, red if enhancing, black if no gadolinium given), / indicates treatment discontinuation. Patient 8 discontinued medication at month 18 but not follow-up from month 18 to 24.

Table 1 Immunologic changes in serum before and after minocycline treatment

	Before	After	<i>P</i> value
IL-12p40 (pg/mL)			
Mean (SEM)	70 (17)	204 (61)	<i>P</i> < 0.01
Median (range)	43 (4–138)	113 (44–618)	
sVCAM-1 (ng/mL)			
Mean (SEM)	795 (72)	1057 (64)	<i>P</i> < 0.01
Median (range)	724 (523–1243)	984 (867–1497)	
sICAM-1 (ng/mL)			
Mean (SEM)	494 (35)	550 (40)	NS
Median (range)	495 (330–667)	556 (404–772)	
MMP-9 content (ng/mL)			
Mean (SEM)	578 (69)	561 (44)	NS
Median (range)	580 (271–910)	597 (353–818)	
TIMP-1 (ng/mL)			
Mean (SEM)	365 (64)	290 (37)	NS
Median (range)	300 (158–725)	275 (137–490)	

Before treatment values refer to results combined from sera obtained at –3 months and time 0 (just before drug initiation), while on-treatment values are pooled from samples collected at 3, 6, 12 and 18 months after minocycline is started. Comparison between the pretreatment and on-treatment groups was tested with the Wilcoxon matched-pairs signed-ranks test. NS, not significant. Statistical significance was defined as less than 0.05.

after month 6, who has reduced IL-12p40 levels after month 6.

Serum IL-12p70, TNF- α , IFN- γ and IL-10 levels were all below the sensitivity of the assay.

Adhesion molecules during minocycline treatment

Treatment increased sVCAM-1 levels compared to pretreatment values (Table 1) but the effect was statistically significant only at 3 months (Table 2). Soluble ICAM-1 was not significantly increased during minocycline treatment (Table 1).

MMP-9 and TIMP-1

The levels of MMP-9 and TIMP-1 in serum were not altered by minocycline (Table 1). However, as

MMP-9 is a protease, its enzymatic activity would be more reflective of its action. Thus, we evaluated sera using a fluorogenic assay based on the capacity of MMP-9 in serum to degrade a substrate that fluoresces upon cleavage. We found that MMP-9 activity was potently reduced by minocycline (Table 1) and that this decrease, reflected in a reduction in V_{max} and K_m , was evident at all post-treatment times examined (Figure 3).

Correlation between MMP-9 activity and IL-12p40

There was an apparent inverse trend between MMP-9 activity and IL-12p40 levels within each patient while on minocycline (data not shown). Indeed, when MMP-9 activity and IL-12p40 levels of all samples were correlated, linear regression analysis showed a significant inverse correlation ($r = -0.83$; $P = 0.039$).

Discussion

Minocycline appears promising as a potential therapy for MS. It is inexpensive, available as oral therapy, and has a long safety record. In addition, tissue culture and animal studies have shown that it has multiple immunomodulatory and neuroprotective activities [3]. However, other than the six-month MRI report in this cohort of patients [8], there is no clinical or immunological data describing the effect of chronic minocycline therapy on MS patients. In this study, we appear to demonstrate benefit on clinical and MRI outcomes in RRMS patients during 24 months of minocycline treatment. In addition, we report immunomodulatory changes in the sera of patients on minocycline therapy that are likely desirable in the control of MS pathophysiology.

This is a pilot study to evaluate the safety of minocycline on MS and to explore the effect of treatment on clinical and MRI parameters to determine if further efficacy trials are indicated. Treatment was tolerated, as expected, based on the

Table 2 Serum concentrations of IL-12 p40 and sVCAM-1 before, and at different times after minocycline

	Pretreatment	On-treatment	<i>P</i> value (Friedman's test)	<i>P</i> value
IL-12p40		3 months	185 (50)	0.001
Mean (SEM)	70 (17)	6 months	195 (59)	0.002
		12 months	208 (78)	0.0078
		18 months	229 (78)	0.0039
sVCAM-1	795 (72)	3 months	1057 (68)	0.044
Mean (SEM)		6 months	933 (102)	0.2754
		12 months	1068 (134)	0.0781
		18 months	1274 (269)	0.1094

Repeated measures non-parametric ANOVA (Friedman's test) was done for within-group comparison ($P < 0.05$). Wilcoxon matched-pairs signed-ranks as post-hoc analysis ($P < 0.01$).

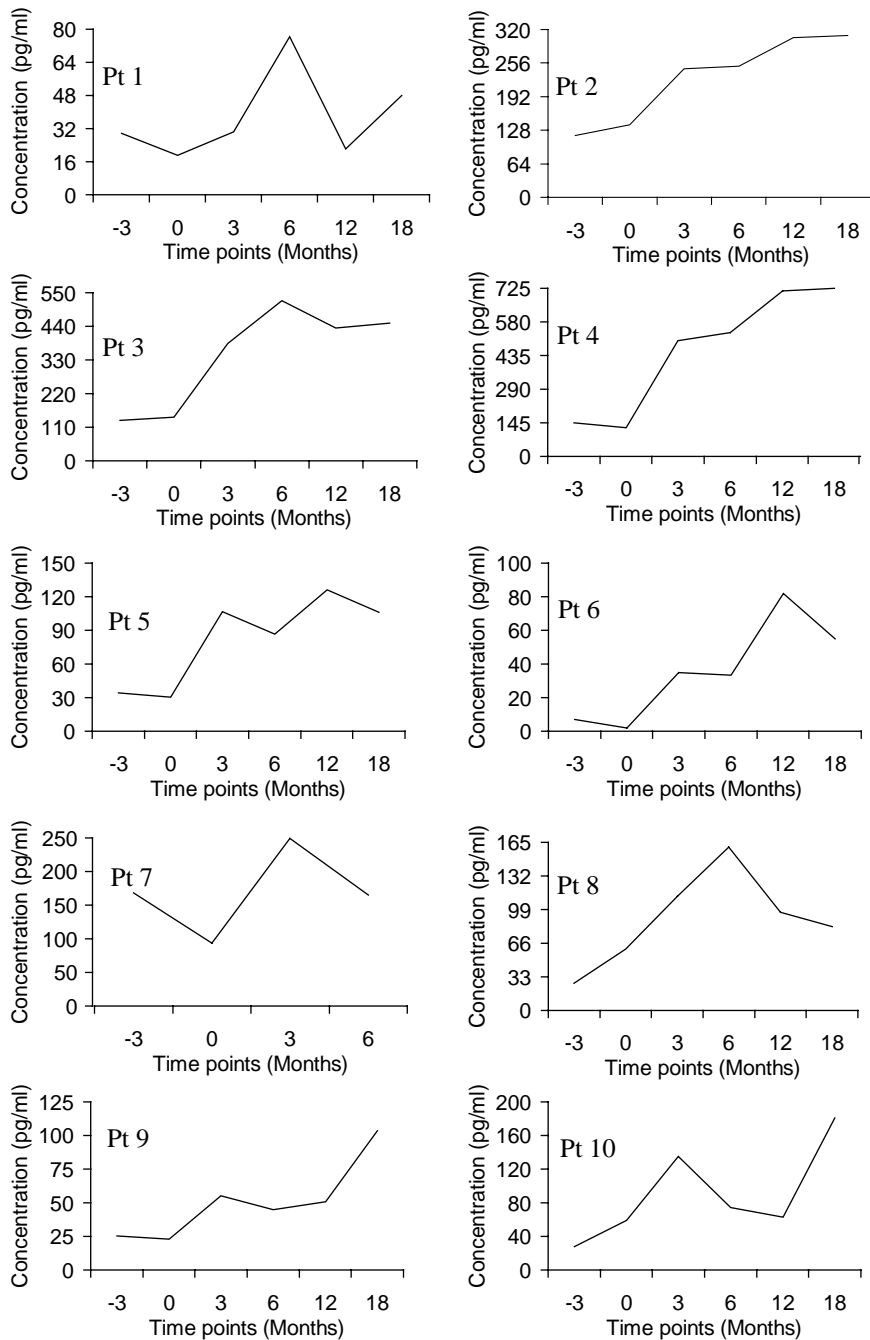


Figure 2 Change of IL-12p40 over time for each patient. Values are in pg/mL. Patient 1 was on half-dose minocycline because of nausea between months 6 and 24. Patient 7 stopped the treatment at month 6.

known side-effect profile of minocycline; nausea and mild dizziness, particularly early in treatment, are common. There was no evidence of disease-specific safety issues; in particular, MRI gadolinium-enhancing activity, the most sensitive outcome measure used to evaluate brain inflammation in MS trials, was not increased in any of the patients. Finally, while conclusions are limited by

the small sample size, both the clinical and the MRI outcomes continue to be promising. The delayed reduction of relapse activity until after month 6 may be related to the phenomenon known as regression to the mean. Because MS is a cyclic disease with periods of greater and lesser activity, and because all patients were selected for having active disease, they may have all naturally moved

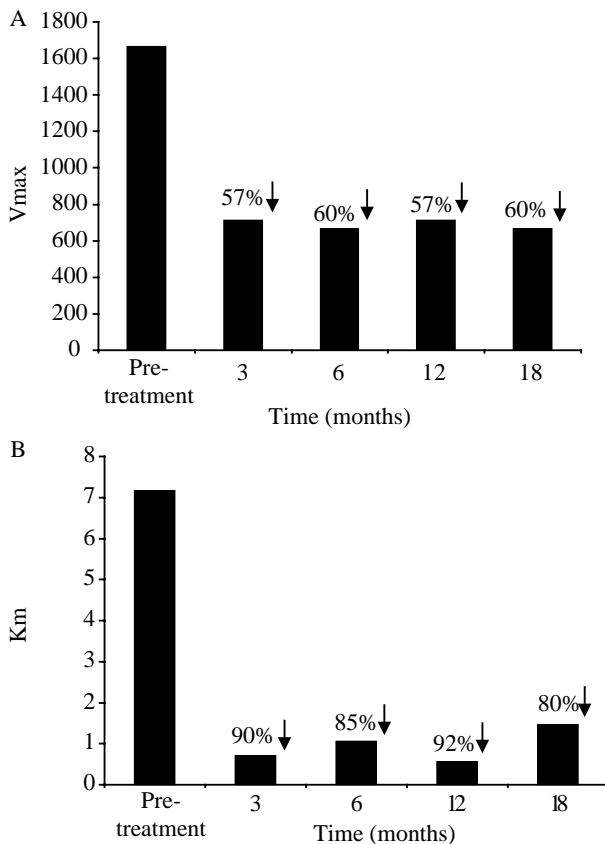


Figure 3 Minocycline decreased the activity of MMP-9 as manifested by the reduction of V_{max} (A, $\mu\text{mol/g per min}$) and K_m (B, μM) on treatment compared to pretreatment values. The magnitude of the decrease at the various time points on treatment is indicated above each bar.

towards a period of less disease activity. Therefore, the resolution of all relapses after month 6 may have been due to this phenomenon. The delayed response on relapses however is not typical of a placebo effect, which is usually early. The rapid and marked decrease in gadolinium enhancing, and the sustained benefit in all patients treated with full-dose therapy, however, is more suggestive of a treatment benefit. Furthermore, it seems unlikely that a placebo effect would lead to reduced sub-clinical activity (MRI) without a reduction in clinical activity (relapses). This potential benefit is further supported by changes in immune biomarkers in this study.

We found that the average peak (2-h post-oral intake) serum level of minocycline in this group of MS patients was about $5 \mu\text{g/mL}$ (unpublished data). This concentration is correspondent with that used to achieve biological effects of minocycline in tissue culture experiments [3].

There was a marked and sustained increase in IL-12p40 during minocycline treatment. The loss

of this increase at 12 and 18 months in the one patient (patient 1) who decreased her dose by 50% and had recurrence of gadolinium enhancement on MRI even suggests there might be a dose effect. IL-12 is a cytokine important in the differentiation of precursor CD4^+ T cells into the proinflammatory T helper 1 (Th1) subset, which is thought to be important in MS pathogenesis [12,13]. IL-12 is a heterodimer composed of two subunits, p40 and p35, which form the mature IL-12p70 cytokine. At first glance, therefore, an elevation of IL-12p40 by minocycline would be considered proinflammatory and detrimental in MS. However, levels of IL-12p40 and IL-12p70 are uncoupled, as IL-12p40 is synthesized in excess of IL-12p70 by 5–500-fold [14]. This excess IL-12p40 can antagonize IL-12, thereby decreasing the effect of IL-12 [15]. One explanation is that the IL-12 receptor is made up of both IL-12R β 1 and β 2. Signal transduction is mediated only through the β 2 component [16], IL-12 p40 binds only to IL-12R β 1 but fails to transduce any intracellular signal when the IL-12R β 2 unit is not engaged. Also, excess IL-12p40 can homodimerize and form IL-12p80, which binds to IL-12R β 1 with high enough affinity to prevent IL-12p70 from binding to the IL-12 receptor [17].

That excess IL-12p40 antagonizes IL-12p70 signalling has also been shown in functional studies [17,18]. Ling *et al.* demonstrated that the IL-12p40 monomer or homodimer binds to IL-12R and inhibits the activation of human lymphoblasts [17]. Also, while IL-12p70 enhances the encephalitogenicity of myelin-specific T cells that were adoptively transferred in mice, the adoptive transfer of IL-12p40-transduced myelin-specific T cells results in less severe clinical disease and fewer relapses [19].

The finding that IL-12p40 is increased in our patients is thus noteworthy. It suggests that minocycline increases the serum concentration of the p40 subunit, which can then potentially antagonize the IL-12p70 molecule. IL-12p40 was elevated in a cohort of primary progressive MS patients after 3 months of treatment with IFN- β 1b [20]. The mean baseline IL-12p40 level was similar in that study ($64\text{--}66.5 \text{ pg/mL}$) to ours (70.5 pg/mL), but following treatment with IFN- β , IL-12p40 increased to only $95.3\text{--}105.3 \text{ pg/mL}$ [20] in contrast to an increase to 204 pg/mL after minocycline treatment in our study.

There is currently an ongoing phase I trial of antibodies to IL-12p40 in RRMS [21]. This treatment could be beneficial if the neutralization of IL-12p40 decreases the activity of the mature IL-12p70 molecule; however, undesirable effects may occur if the putative benefits of IL-12p40, as described above, are interfered with. In support,

an IgG2b-IL-12p40 fusion protein had a beneficial effect in another immune-mediated disorder, Crohn's disease, when used at low concentrations, but a detrimental effect when used at high concentrations [22]. Finally, IL-12p40 is also a subunit of another cytokine, IL-23, and of the cytokine receptors IL-6R and ciliary neurotrophic factor receptor (CNTFR) [16]. The impact of minocycline and IL-12p40 antibodies on IL-23 and these cytokine receptors remains unknown at this point.

Measurement of adhesion molecules provides further insight into the mechanisms of minocycline in RRMS. VCAM-1 is an adhesion molecule found on glial cells and vessels, including the microvessels of the brain. Interaction of VCAM-1 with the integrin VLA-4 facilitates leukocyte entry into the CNS [23]. VCAM-1 exists as both membrane (m)-bound and soluble forms. The membrane-bound form is upregulated in different stages of acute inflammation. The soluble form is proteolytically cleaved from the membrane-bound form. There is an increased level of sVCAM-1 in the sera and CSF of MS patients during acute disease. However, the increase is not thought to be the cause of the disease activity as sVCAM-1 levels are increased during treatment with IFN- β and correlate with a decrease in gadolinium-enhancing lesions [24]. Indeed, sVCAM-1 is thought to exert negative feedback on inflammation as it binds to VLA4 integrin on inflammatory cells, hindering the interaction of these cells with the blood-brain barrier [25]. The increase in sVCAM-1 by minocycline is a novel mechanism of this drug. It is unclear if it is only an early phenomenon because while the increase was only statistically significant at 3 months, the mean level continued to increase. Further studies and a larger sample size are needed to address this issue.

MMPs are implicated in several steps in the pathogenesis of MS, including the transmigration of leukocytes into the parenchyma of the CNS, promotion of neuroinflammation, and neurotoxicity [26]. Indeed, a mechanism that may account for the efficacy of IFN- β in MS is its reduction of the production of MMP-9 by T cells, reducing their influx into the CNS [27–30]. In the current study, we found no evidence of alterations of levels of MMP-9 or TIMP-1. However, enzyme content does not necessarily equate with proteolytic activity and, indeed, minocycline treatment decreased MMP-9 activity in serum. The apparent reduction of V_{max} and K_m indicates that minocycline is acting upon MMP-9 in serum as an uncompetitive inhibitor, therefore decreasing the maximum velocity of the reaction and increasing the affinity of MMP-9 to the substrate [11]. An uncompetitive inhibitor binds outside the active site of the enzyme. Binding

of the inhibitor occurs as the substrate-enzyme complex forms, where induced conformational rearrangements expose binding sites that are not normally present under basal, non-reactive conditions. Upon formation of the minocycline-enzyme complex, a permanent rearrangement of the tertiary structure of the enzyme can occur. This may result in permanent loss of activity. MMPs contain a zinc ion in the catalytic site. In addition, many MMPs contain a second zinc atom which, like Ca^{2+} , is believed to confer structural stability to the enzyme. It has long been known that cation chelators such as EDTA (which chelates both Ca^{2+} and Zn^{2+}) or 1,10-phenanthroline (which binds only Zn^{2+}) can inactivate MMPs *in vitro*. Tetracycline, minocycline and doxycycline have affinity for divalent cations such as zinc (Zn^{2+}) [31]. Thus, it is possible that minocycline is interacting with the Zn^{2+} ion responsible for conferring structural stability to MMP-9, resulting in an overall, permanent loss of functionality. It is also conceivable that the effect of minocycline on MMP-9 activity is indirect, through mechanisms that are not clear at this point.

The finding of a statistically significant inverse correlation between MMP-9 activity and IL-12p40 is noteworthy. It is uncertain if this indicates that MMP-9 activity regulates IL-12p40, or vice versa. Alternately, the reduction of MMP-9 activity and the elevation of IL-12p40 may not be linked mechanistically. They may even be additive in their impact on decreasing neuroinflammation.

Orally available statins have gained attention as potential treatments in MS. In this regard, lovastatin was found to reduce MRI enhancing activity by 43% and to effect a small reduction in relapse rate at 12 months [32]. Simvastatin decreased enhancing lesions by 44% after 6 months but did not affect relapses [33]. Arguably, the results of minocycline herein are of a larger magnitude than those found for statins. However, it is prudent to be cautious when comparing across trials as methodological differences and study population could influence outcomes.

In conclusion, this study shows that treatment with minocycline was associated with promising clinical outcomes. Because there is no placebo group, it is unclear if the marked decrease in relapse activity is due to the effect of treatment, or due to chance, regression to the mean or a placebo effect. The concurrent and complete absence of MRI activity in patients receiving full-dose minocycline, and the changes in several biological markers, however, argue in favour of a treatment impact due to minocycline. The results support the further investigation of minocycline in MS.

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