

# Targeting ASIC1 in primary progressive multiple sclerosis: evidence of neuroprotection with amiloride

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Neurodegeneration is the main cause for permanent disability in multiple sclerosis. The effect of current immunomodulatory treatments on neurodegeneration is insufficient. Therefore, direct neuroprotection and myeloprotection remain an important therapeutic goal. Targeting acid-sensing ion channel 1 (encoded by the *ASIC1* gene), which contributes to the excessive intracellular accumulation of injurious  $\text{Na}^+$  and  $\text{Ca}^{2+}$  and is over-expressed in acute multiple sclerosis lesions, appears to be a viable strategy to limit cellular injury that is the substrate of neurodegeneration. While blockade of ASIC1 through amiloride, a potassium sparing diuretic that is currently licensed for hypertension and congestive cardiac failure, showed neuroprotective and myeloprotective effects in experimental models of multiple sclerosis, this strategy remains untested in patients with multiple sclerosis. In this translational study, we tested the neuroprotective effects of amiloride in patients with primary progressive multiple sclerosis. First, we assessed ASIC1 expression in chronic brain lesions from post-mortem of patients with progressive multiple sclerosis to identify the target process for neuroprotection. Second, we tested the neuroprotective effect of amiloride in a cohort of 14 patients with primary progressive multiple sclerosis using magnetic resonance imaging markers of neurodegeneration as outcome measures of neuroprotection. Patients with primary progressive multiple sclerosis underwent serial magnetic resonance imaging scans before (pretreatment phase) and during (treatment phase) amiloride treatment for a period of 3 years. Whole-brain volume and tissue integrity were measured with high-resolution  $T_1$ -weighted and diffusion tensor imaging. In chronic brain lesions of patients with progressive multiple sclerosis, we demonstrate an increased expression of ASIC1 in axons and an association with injury markers within chronic inactive lesions. In patients with primary progressive multiple sclerosis,

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we observed a significant reduction in normalized annual rate of whole-brain volume during the treatment phase, compared with the pretreatment phase ( $P = 0.018$ , corrected). Consistent with this reduction, we showed that changes in diffusion indices of tissue damage within major clinically relevant white matter (corpus callosum and corticospinal tract) and deep grey matter (thalamus) structures were significantly reduced during the treatment phase ( $P = 0.02$ , corrected). Our results extend evidence of the contribution of ASIC1 to neurodegeneration in multiple sclerosis and suggest that amiloride may exert neuroprotective effects in patients with progressive multiple sclerosis. This pilot study is the first translational study on neuroprotection targeting ASIC1 and supports future randomized controlled trials measuring neuroprotection with amiloride in patients with multiple sclerosis.

**Keywords:** multiple sclerosis; neuroprotection; acid-sensing ion channel; MRI; amiloride

**Abbreviation:** EDSS = Expanded Disability Status Scale

## Introduction

Multiple sclerosis is the major neurological cause of progressive disability in young adults, in which inflammatory demyelination within the CNS is associated with various degrees of neurodegeneration that may occur throughout the disease course (Ferguson *et al.*, 1997; Rocca *et al.*, 2003; De Stefano *et al.*, 2010). The extent of neuroaxonal loss correlates with clinical impairment (De Stefano *et al.*, 1998; Bjartmar *et al.*, 2000) and forms the pathophysiological substrate of permanent disability (Tallantyre *et al.*, 2010). Current treatments focusing on reduction of inflammation exert only an indirect effect on neurodegeneration, with limited impact on clinical disability in relapsing remitting disease and no effect on the primary progressive or non-relapsing secondary progressive phase (Leary *et al.*, 2003; Wolinsky *et al.*, 2007; Hawker *et al.*, 2009; Montalban *et al.*, 2009). Therefore, the development of primary neuroprotective strategies remains a major therapeutic aim.

Cellular damage and neurodegeneration in the CNS has been closely linked to the activation of injurious cellular cascades through excess accumulation of intra-axonal  $\text{Na}^+$  and  $\text{Ca}^{2+}$  ions (Stys and Lopachin, 1998; Waxman, 2008). Whilst mechanisms of  $\text{Na}^+$  and  $\text{Ca}^{2+}$  influx are multifactorial, voltage-gated sodium channels have been shown to be an important, albeit not exclusive, contributory component (Nikolaeva *et al.*, 2005). Neuroprotective efficacy of voltage-gated sodium channel blockade has been demonstrated in CNS injury (Fern *et al.*, 1993) and multiple sclerosis models (Lo *et al.*, 2002; Bechtold *et al.*, 2012) but has not clearly translated to patients with secondary progressive multiple sclerosis (Kapoor *et al.*, 2010). However, more recent evidence suggests that cellular protection can be exerted through blockade of the neuronal proton-gated acid-sensing ion channel 1 (ASIC1), which is increased within axons and oligodendrocytes in acute multiple sclerosis lesions (Vergo *et al.*, 2011). The inflammatory 'milieu' in multiple sclerosis provides a permissive environment to facilitate ASIC1 opening and conductance of  $\text{Na}^+$  and  $\text{Ca}^{2+}$ . Blocking ASIC1 with amiloride exerts neuroprotective and myeloprotective effects in acute and chronic experimental models of multiple sclerosis (Friesse *et al.*, 2007; Vergo *et al.*, 2011). The neuroprotective and myeloprotective effects of amiloride occur independently from any significant anti-inflammatory effect of the drug, as previous studies have not demonstrated any significant

influence of amiloride on the immunological component of CNS inflammation (Friesse *et al.*, 2007; Vergo *et al.*, 2011). Moreover, the protective effect occurs downstream of inflammation and remains evident even when administered after the onset of inflammation in an animal model of multiple sclerosis (Friesse *et al.*, 2007; Vergo *et al.*, 2011).

In this study, we tested the neuroprotective effects of amiloride in patients with primary progressive multiple sclerosis. This group of patients was selected as natural history studies demonstrate the rate and character of the progressive phase are similar between secondary progressive and primary progressive multiple sclerosis cohorts (Kremenchtzky *et al.*, 2006). Furthermore, neuropathological studies indicate a greater predilection to chronic inactive lesions with primary progressive and secondary progressive multiple sclerosis compared with active lesions in relapsing remitting multiple sclerosis (Kutzelnigg *et al.*, 2005), and therefore, any positive effect on outcome would support the hypothesis of a direct neuroprotective effect. In addition, owing to the lack of efficacy, patients with primary progressive multiple sclerosis are immunotherapy treatment naïve, thus avoiding any confounding treatment effects.

First, we examined ASIC1 expression in chronic brain lesions from post-mortem of progressive patients with multiple sclerosis (Study 1, *ex vivo*) to detect the presence of neurodegenerative molecular signature amenable to amiloride blockade in progressive multiple sclerosis. Second, we tested the neuroprotective effect of amiloride in a cohort of 14 patients with primary progressive multiple sclerosis (Study 2, *in vivo*) using MRI markers of neurodegeneration as outcome measures of neuroprotection (Barkhof *et al.*, 2009). Patients with primary progressive multiple sclerosis underwent serial MRI scans, including high-resolution  $T_1$ -weighted and diffusion-weighted imaging over a period of 3 years, before (pretreatment phase) and during (treatment phase) amiloride treatment. We tested the rate of change in MRI outcome measures during the pretreatment compared with the treatment phase under the hypothesis that significant between-phase changes reflected a neuroprotective effect of amiloride in patients. We used a whole-brain atrophy measure that is the current gold standard for measuring neurodegeneration longitudinally in clinical trials as the primary outcome (Smith *et al.*, 2002; Barkhof *et al.*, 2009). Integrity of remaining brain tissue, measured using diffusion tensor imaging, was used to capture significant changes in

neurodegenerative processes, which are reflected in altered brain microstructural architecture (Alexander *et al.*, 2007).

Whilst extending our current knowledge on the basic neuroscience of neurodegenerative mechanisms, this study contributes to the translational efforts supporting the development of therapeutic strategies for neuroprotection in multiple sclerosis. Other neurodegenerative conditions may also benefit from these findings.

## Materials and methods

### Study 1: *ex vivo*

#### Immunohistochemistry

Post-mortem spinal cord tissue acquired from patients with progressive multiple sclerosis ( $n=6$ ,  $63 \pm 6.5$  years, mean disease duration:  $25 \pm 5.6$  years) and from healthy control subjects ( $n=5$ ,  $74 \pm 5.6$  years) with no CNS disease was obtained from the NeuroResource tissue bank, University College of London Institute of Neurology, London, UK (Table 1).

The analysed tissue was rapidly frozen (post-mortem delay:  $16 \pm 1.7$  h) as  $1 \text{ cm}^3$  blocks on Tissue-Tek O.C.T. mounting medium. Characterization of the lesions was performed using oil red O and haematoxylin staining to identify the inflammatory activity within the lesion. Chronic inactive multiple sclerosis lesions were identified by demyelination and the presence of low number of oil red O-positive macrophages. For ASIC1 immunohistochemistry, the snap-frozen sections ( $10 \mu\text{m}$ ) were fixed for 10 min in acetone, permeabilized in PBS containing 0.1% Triton<sup>TM</sup> X-100, and endogenous peroxidase activity was quenched by incubating the sections in 3%  $\text{H}_2\text{O}_2$  before incubating in blocking solution (PBS containing 5% normal goat serum and 3% bovine serum albumin). Anti-mouse/human polyclonal antiserum (MTY19) recognizing ASIC1 (Wemmie *et al.*, 2003) and other antibodies against intermediate neurofilaments (NF68, Covance), amyloid- $\beta$  precursor protein (MAB348, Millipore), myelin

basic protein (MBP) (SMI-94, Covance) and 2',3'-cyclic nucleotide 3'-phosphodiesterase (CNPase) (MAB326R, Millipore) were incubated overnight at  $4^\circ\text{C}$  in blocking solution containing 0.1% Triton<sup>TM</sup> X-100. Tissue sections were washed in PBS and incubated with secondary goat anti-mouse IgG-Alexa Fluor<sup>®</sup> 488 (1:1000; Molecular Probes), horseradish peroxidase-goat anti-rabbit IgG (1:100) and Alexa Fluor<sup>®</sup> 568 tyramide according to manufacturer's recommendation (TSA Fluorescence Systems, Molecular Probes, Invitrogen). Tissue sections were washed in PBS and counterstained with DAPI  $1 \mu\text{g}/\text{ml}$  before mounting (Dako, fluorescence mounting media). All incubations were performed at room temperature unless otherwise stated. Images were captured by Lasershar software (Zeiss) and a confocal system on a microscope (LSM510; Zeiss) coupled with a high-resolution digital camera. Axonal quantification (multiple sclerosis, 1985 axons; control, 985 axons) was performed using an adapted methodology as previously described (Vergo *et al.*, 2011) and statistical analysis performed with Fishers exact test.

### Study 2: *in vivo*

This study was approved by the Oxford Research Ethics Committee (ethics no. 08/H0604/155).

#### Participants and study design

Patients with primary progressive multiple sclerosis according to the revised McDonald criteria (Polman *et al.*, 2005) were recruited from the Oxford multiple sclerosis service, John Radcliffe Hospital, Oxford, UK. Patients were eligible if immunomodulatory treatment naïve.

This open-label 3-year study included a pretreatment and a treatment phase (Fig. 1). MRI scans were performed at five time points before treatment (pretreatment): dual scans, i.e. MRI scan 2 weeks apart, at onset (T1 and T2) and 12 months later (T3 and T4). In addition, a fifth (single) scan (T5) was performed later just before an MRI scanner upgrade (at an interval of 5–15 months). After the upgrade, the treatment phase started with dual scans performed again just before starting amiloride (T6 and T7) and then 12 months later, at the end of the study (T8 and T9). The final dual scans were performed 2 weeks after stopping amiloride to prevent the diuretic effect of the drug affecting the brain volume measure. Clinical assessments were performed using the Expanded Disability Status Scale (EDSS) (Kurtzke, 1983) at baseline, 1 year, T5 and at baseline and after 1 year in the treatment phase [Consolidated Standards of Reporting Trials (CONSORT) flow diagram; Fig. 2].

#### Drug intervention

Amiloride was given orally at a daily dosage of 10 mg, once the scan T7 had been performed. It was continued for 1 year and stopped 2 weeks before scan T8. Although amiloride is already licensed for other indications and possesses a known safe profile of side effects, side effects were recorded by means of a diary during the study. Blood tests for urea and electrolytes were performed at  $-4$ , 0, 4, 24 and 52 weeks after the commencement of amiloride. Compliance was ensured, and adverse events checked through regular telephone contact and medication diary.

#### Imaging

##### Magnetic resonance imaging acquisition

Brain scans were obtained on a 1.5 T Siemens Sonata magnetic resonance scanner at each visit. We acquired  $T_1$ -weighted 3D Ultra fast Gradient echo sequence (repetition time = 2600 ms, echo time = 5 ms,  $T_1 = 850$  ms; voxel size =  $1 \times 1 \times 1.2$  mm) for volumetric data. For

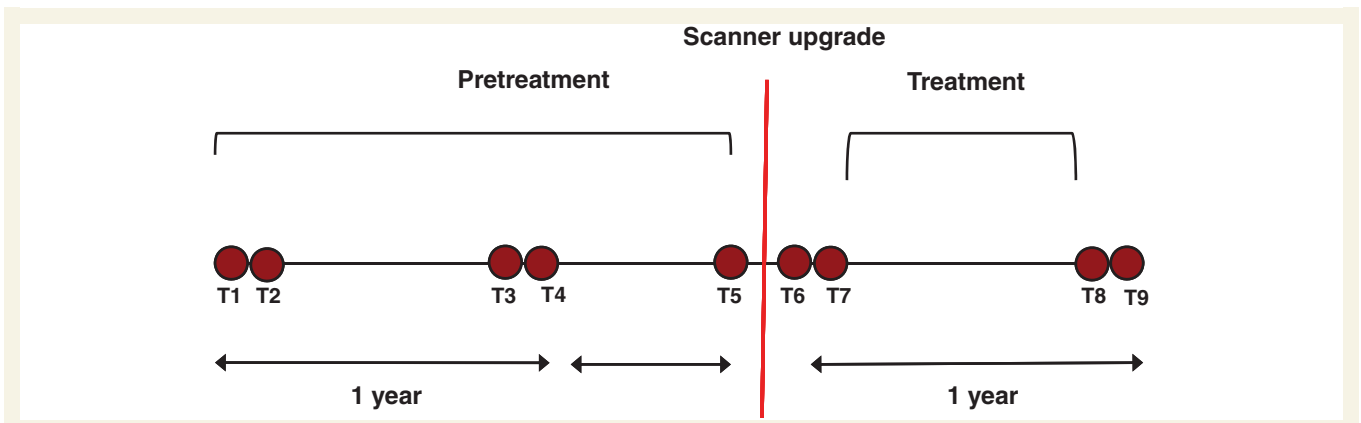
**Table 1** Demographic and neuropathological data for the cases with multiple sclerosis

Case/age/sex	Oil red O score <sup>a</sup>	Type of disease	Disease duration	Post-mortem delay (h)
MS1/51/M	1, 2	SP	20	8
MS2/59/F	0, 1	SP	20	13
MS3/69/M	0, 1	SP	21	14
MS4/62/M	1, 1	SP	28	15
MS5/64/M	1, 1	SP	31	17
MS6/71/F	1, 1	SP	32	19
CON1/81/M	–	NA	NA	19
CON2/72/M	–	NA	NA	20
CON3/79/F	–	NA	NA	21
CON4/75/F	–	NA	NA	7
CON5/65/M	–	NA	NA	24

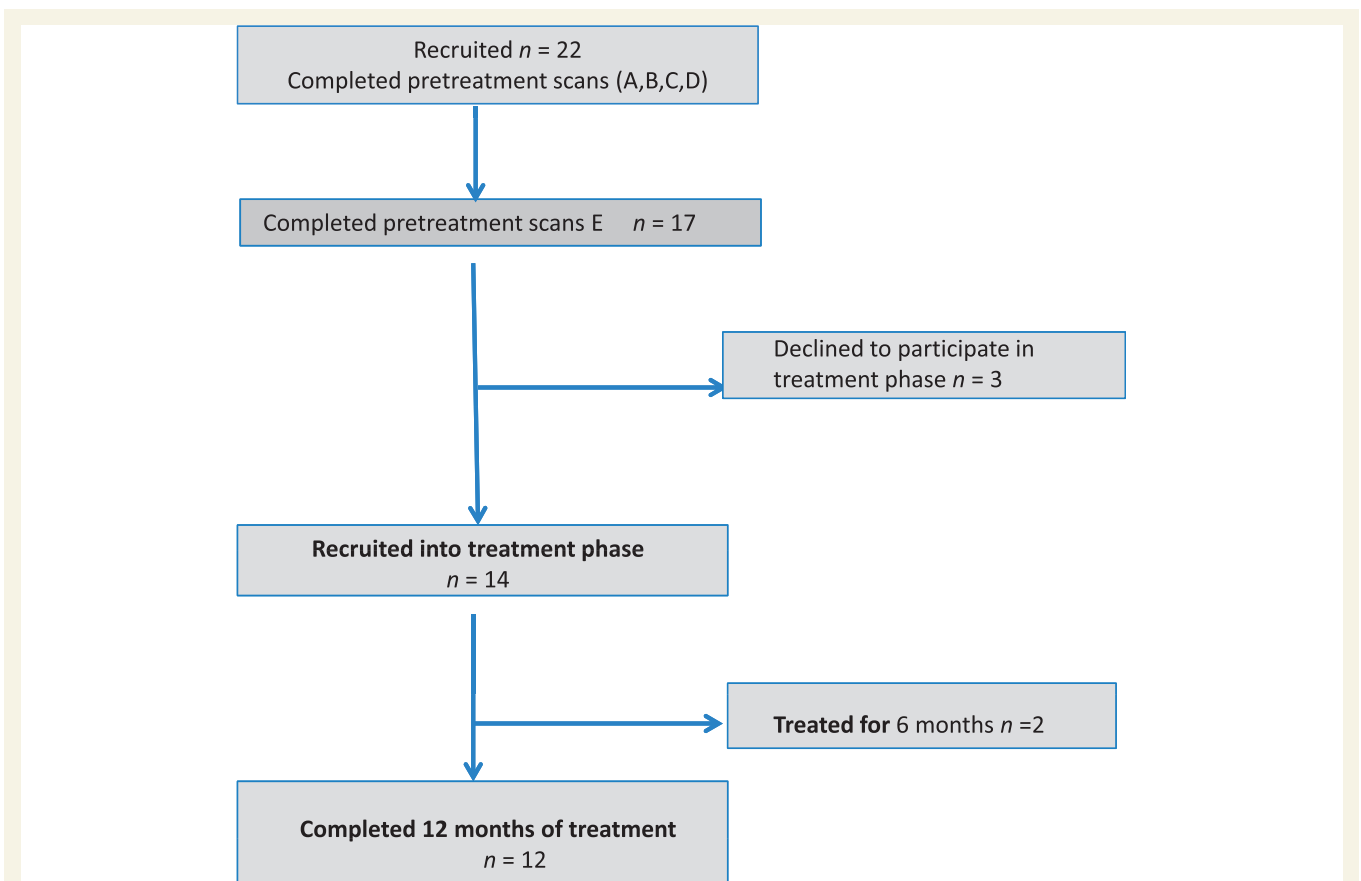
<sup>a</sup> First digit shows the score for number of oil red O-positive macrophages containing lipids resulting from myelin breakdown on a scale from 0 to 5. The second digit is the score for peri-venular inflammatory cuffing obtained from haematoxylin staining on a scale from 0 to 5.

Age and disease duration are indicated in years.

CON = control; F = female; M = male; MS = multiple sclerosis; NA = not applicable; SP = secondary progressive.



**Figure 1** Study design. Time points for MRI scanning are shown as filled circles. Dual scans (e.g. T1 and T2) were separated by an interval of 2 weeks.



**Figure 2** Consort flow diagram demonstrating patient flow through the pretreatment phase (A–E) and amiloride treatment phase.

diffusion tensor imaging, pulsed spin-echo planar sequences (PGSE, repetition time = 8600 ms, echo time = 83 ms, slice thickness = 2.5 mm) with diffusion gradients were applied in 12 non-collinear directions, with two b-factors ( $b_1 = 0$  and  $b_2 = 1000 \text{ s/mm}^2$ ) and isotropic voxel size of  $2.5 \times 2.5 \times 2.5 \text{ mm}$ . Two sets of diffusion-weighted images were obtained. Field maps measuring the  $B_0$  field deviations were acquired to correct for spatial distortions in the diffusion tensor imaging data. Both the volumetric and diffusion images were acquired at each time point except for T5 of the pretreatment phase, when only the volumetric data were acquired.

**Magnetic resonance data analysis**

Image analysis was carried out using tools from the FMRIB Software Library (FSL, [www.fmrib.ox.ac.uk/fsl](http://www.fmrib.ox.ac.uk/fsl)). The imaging measures were assessed in random order by researchers blinded to the patient’s clinical data and treatment phase.

**Whole-brain volume analyses**

High-resolution  $T_1$ -weighted imaging was used to quantify whole-brain volumes. Percentage whole-brain volume change during the study phases was obtained using SIENA, a robust and highly

reproducible method to quantify changes of brain tissue boundary location over time.

### Diffusion image analysis

Diffusion tensor imaging data were processed using the fuzzy distance transform and tract-based spatial statistics pipeline as described in <http://www.fmrib.ox.ac.uk/fsl/tbss/index.html> (Smith *et al.*, 2006). Diffusion tensor MRI metrics were calculated across the brain and mean diffusivity, axial diffusivity and radial diffusivity. Tract-based spatial statistics project each subject's data onto a mean white matter skeleton to avoid partial volume effect. Average values of the diffusion tensor imaging metrics in the corpus callosum and the corticospinal tract (Fig. 3) within the tract-based spatial statistics white matter skeleton were calculated because these values could reflect the pathological changes underlying disease progression in primary progressive multiple sclerosis (Bodini *et al.*, 2011). These were identified using the Jülich histological atlas (</fsl/data/atlas-descriptions.html>).

To quantify changes in deep grey matter structures, the thalamus was selected as a further region of interest analysis by manually segmenting the right and left thalami in native space, and the mean mean diffusivity extracted for each subject (Fig. 3).

### Estimation of rates of changes in brain volume and tissue integrity: modelling imaging outcomes

To test the neuroprotective effect of ASIC1 blockage, we compared the rate of changes in the pretreatment versus amiloride treatment phases.

We converted the per cent brain volume change estimates resulting from SIENA analysis taken between the pretreatment time points T1–T3, T2–T4 and T3–T5, and the post-treatment time points T6–T8 and T7–T9 into annual rates of change of whole-brain volume by dividing by the corresponding time interval.

We applied a general linear model for each measure to estimate the mean annual rates of change in the pretreatment and treatment

phases. This was achieved by formulating one general linear model that pooled the SIENA estimates (to calculate the mean rates) and a separate general linear model that estimates the appropriate linear slopes and intercepts for the diffusion tensor imaging measures (taking individual values from all time points, except T5, as diffusion tensor imaging was not measured in this session). Both general linear models also calculate the differences between the mean annual rates of change, along with the corresponding variance in this estimate, driven by the variability in the repeated measurements (where repeated measurements refers to measures such as T2 and T1 for diffusion tensor imaging or T1–T3 and T2–T4 for SIENA).

The upgraded scanner was from the same manufacturer with equivalent field strength as the pre-upgrade scanner, and standardization was performed using healthy control subjects and phantoms. To ensure that the effect of the upgrade on the individual rates of change was minimized, the pretreatment phase was completed before the upgrade, and the amiloride treatment phase was started after the upgrade.

### Statistical testing

The statistical analyses used quantities that were normalized for individual subject variability, by using a test statistic equal to the ratio of the difference in annualized rates of change (between amiloride treatment and pretreatment phases) to the corresponding standard deviation (SD) of this difference in rates, as given by the general linear model fitting. That is, the test statistic = (mean amiloride treatment phase rate of change – mean pretreatment phase rate of change) / (SD of the above difference). Statistical testing was performed on this contrast-to-SD ratio, separately for each imaging measure, using a non-parametric test. This test was performed using the Randomise tool in FSL which is an implementation of a permutation-based non-parametric inference method (Nichols and Holmes, 2002). The result of each permutation test was a single uncorrected *P*-value under the null hypothesis that the average pretreatment and treatment rates of change were equal.

Since two hypotheses were being tested (SIENA and diffusion tensor imaging can detect treatment effects), all *P*-values were corrected for multiple comparisons using a Bonferroni correction.

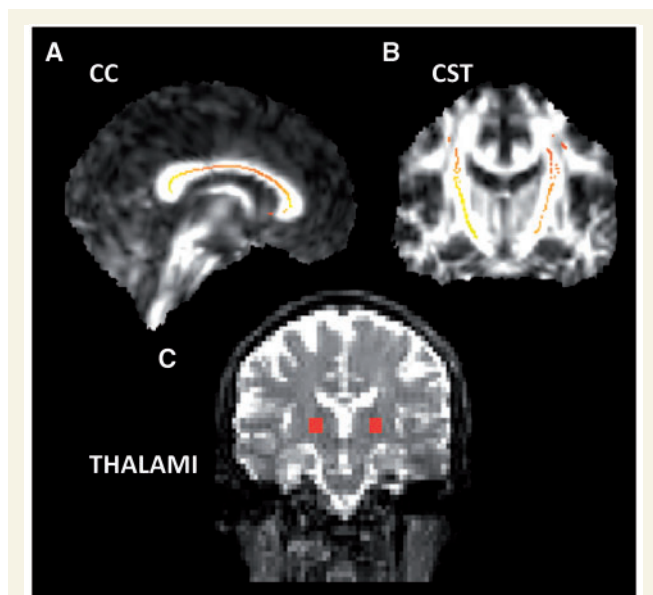
An omnibus test over all the independent diffusion measurements (axial diffusivity and radial diffusivity for corpus callosum and corticospinal tract; mean diffusivity for thalamus) was performed to look for a combined effect on brain tissue integrity. The omnibus test was implemented using a Kolmogorov–Smirnov test to compare the empirical distribution of uncorrected *P*-values from the individual permutation tests on the separate diffusion measures with a uniform distribution, since, under the null hypothesis, the *P*-values would be uniformly distributed.

## Results

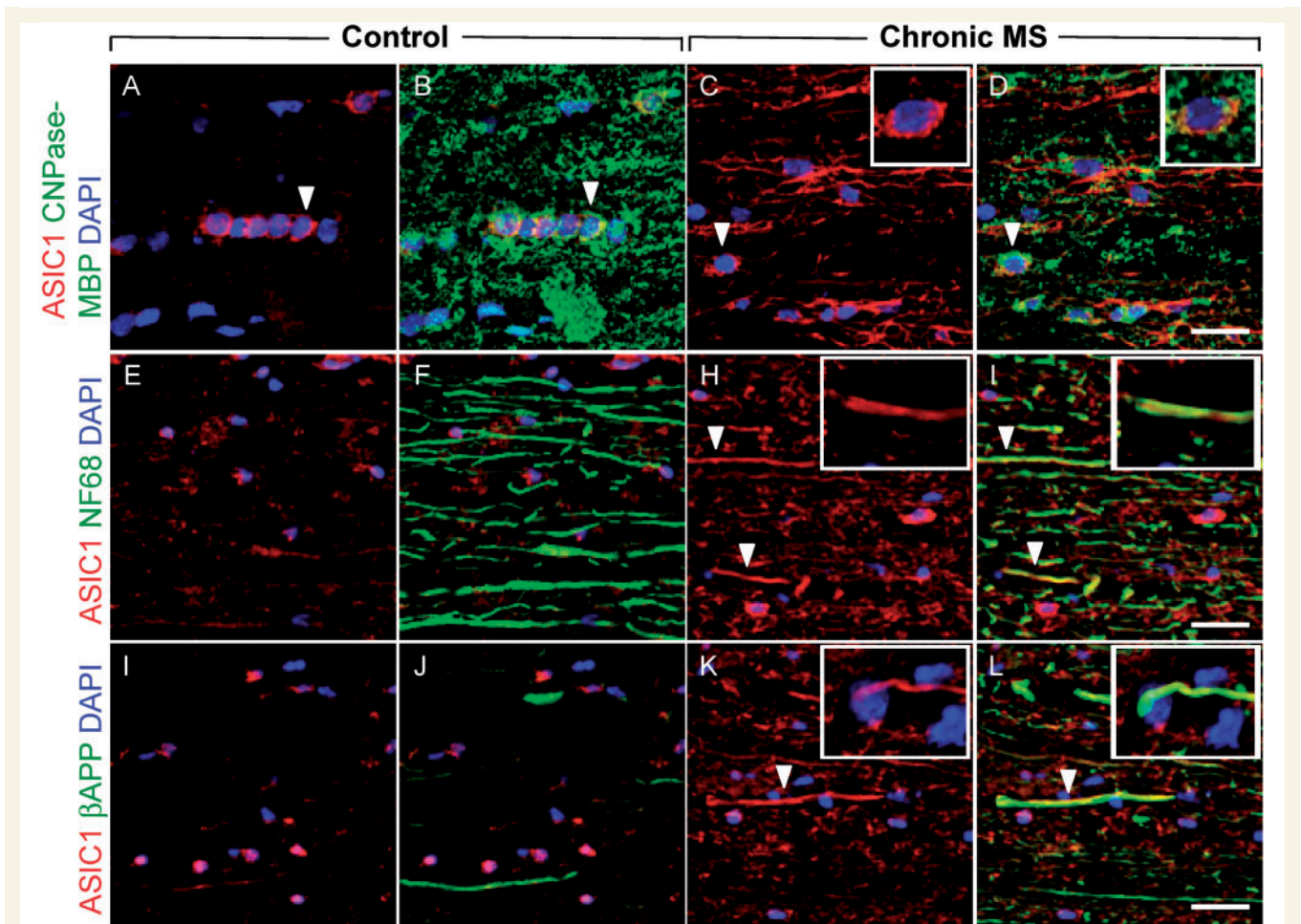
### Study 1: ex vivo

#### Increased expression of ASIC1 in axons and oligodendrocytes in chronic inactive lesions of cases with progressive multiple sclerosis

Compared with healthy controls (15%, 150 of 985 were ASIC positive), significantly more axonal profiles in chronic inactive lesions from multiple sclerosis cases expressed ASIC1 (77%, 1039 of 1339 were ASIC positive) ( $P < 0.001$ ). Consistent with previous



**Figure 3** Demonstrates the three regions of interest selected for the combined diffusion tensor imaging outcomes (colours are arbitrary): (A) corpus callosum (CC), (B) corticospinal tract (CST) and (C) thalamus.



**Figure 4** ASIC1 expression in chronic multiple sclerosis (MS) lesions (A–D). Longitudinal spinal cord section showing ASIC1 expression in the majority of oligodendrocytes both in control and chronic multiple sclerosis lesion. Scale bar = 20  $\mu$ m. (E–H) Axons with intermediate neurofilaments (NF68) demonstrate ASIC1 expression in chronic multiple sclerosis lesions (G and H) but not in control (E and F). Similarly, ASIC1 expression is associated with the axonal injury marker amyloid precursor protein ( $\beta$ -APP) in chronic multiple sclerosis lesions (K and L) but not in control (I and J). Scale bar = 40  $\mu$ m. White arrows indicate example of ASIC1 immunopositive oligodendrocyte and axons.

studies (Vergo *et al.*, 2011) axons with an injury profile (amyloid precursor protein-positive, terminal ovoids and axonal swellings) were frequently (84%, 545 of 646 were ASIC/amyloid precursor protein-positive) seen to co-express ASIC1 (Fig. 4). ASIC1-positive oligodendrocytes were identified in chronic multiple sclerosis lesions, showing a molecular signature that may lead to cellular damage.

## Study 2: *in vivo*

### Patients, drug safety and compliance to study medication

Patients' details are summarized in Table 2. Amiloride was well tolerated in all the patients except for two patients who discontinued treatment after 6 months owing to worsening of their bladder symptoms. In these two patients, dual scans were performed 2 weeks later to allow the rate of change on treatment to be calculated.

**Table 2** Demographic data for patients with primary progressive multiple sclerosis

Item	Median	Range
Age (years)	53.5	41–60
Sex (F/M)	9/5	
Disease duration (years)	6.5	3–18
EDSS pretreatment	4.75	1.5–7
EDSS post-treatment	4.88	1.5–7

### Amiloride slows rates of imaging markers of tissue damage and clinical disability in patients with primary progressive multiple sclerosis

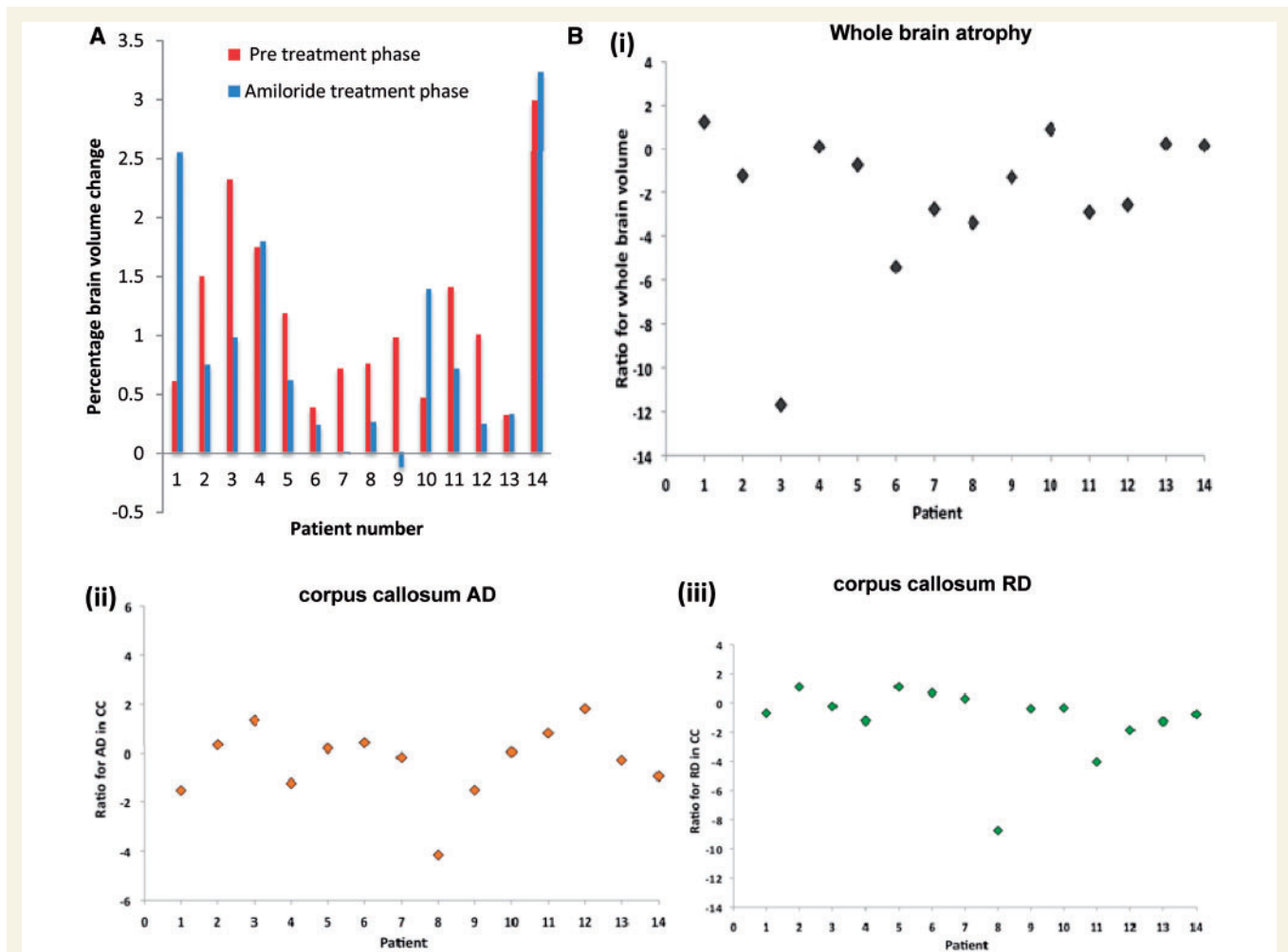
Table 3 shows the unpaired mean values of atrophy and change in diffusion tensor imaging measures for the group as a whole during the pretreatment and treatment phases.

**Table 3** Mean values (SD) of EDSS percentage change/year, atrophy percentage change/year and diffusion tensor imaging measures during the pre-treatment and treatment phases

Item	Pre-treatment (adjusted for time); mean (SD)	On treatment (adjusted for time); mean (SD)
EDSS $\Delta\%$ /year	0.71767 (0.535)	0.25 (0.510)
Atrophy $\Delta\%$ /year	1.16888 (0.88271)	0.92351 (0.99459)
Corpus callosum		
Axial diffusivity	1.4753 (3.3963) <sup>a</sup>	0.49381 (1.8532) <sup>a</sup>
Radial diffusivity	0.66780 (6.8277) <sup>a</sup>	1.0002 (1.3849) <sup>a</sup>
corticospinal tract		
Axial diffusivity	1.1815 (1.9769) <sup>a</sup>	0.63748 (1.0648) <sup>a</sup>
Radial diffusivity	0.84480 (1.2608) <sup>a</sup>	0.10837 (0.90686) <sup>a</sup>
Thalamic mean diffusivity	1713.4 (1840.2) <sup>a</sup>	-57.827 (1650.5) <sup>a</sup>

a  $\times 10^{-5}$ .

To relate the individual pre- and post-treatment rates of change and adjust for the reliability of the measures (variability in the repeated measures), we calculated the differences between the mean annual rates of change and divided by the corresponding variance in this estimate. This showed a significant reduction in the rate of brain atrophy during the amiloride treatment compared with the pretreatment phase ( $P = 0.018$ , corrected) (Fig. 5A and Bi). The rate of change of the combined diffusion measures shown by the omnibus test was significantly reduced during the treatment phase when compared with the pretreatment ( $P = 0.02$ , corrected; individual uncorrected  $P$ -values were 0.23 for axial diffusivity and 0.033 for radial diffusivity in the corpus callosum, 0.20 for axial diffusivity and 0.23 for radial diffusivity in the corticospinal tract and 0.024 for mean diffusivity in the thalamus). The ratios of between-phase difference (defined as the difference in rates divided by the standard deviation of this difference measurement) for each quantity, which take into account the variability in



**Figure 5** (A) Pretreatment and post-treatment atrophy rates for individual patients. (B) Rate of change on treatment minus rate for pretreatment, adjusted for variability (i.e. a ratio formed by dividing the difference in rates by the SD of this difference). Positive values indicate faster rates of changes during the amiloride treatment phase compared with the pretreatment phase, whereas negative values indicate slower rates of change in the amiloride treatment phase compared with the pretreatment phase: (i) brain atrophy, (ii) axial diffusivity (AD) in corpus callosum (CC), (iii) radial diffusivity (RD) in corpus callosum, (iv) axial diffusivity in corticospinal tract (CST), (v) radial diffusivity in corticospinal tract and (vi) mean diffusivity of thalamus. Before statistical testing, an average of the diffusion measures was produced across homologous regions of the two hemispheres.

(continued)

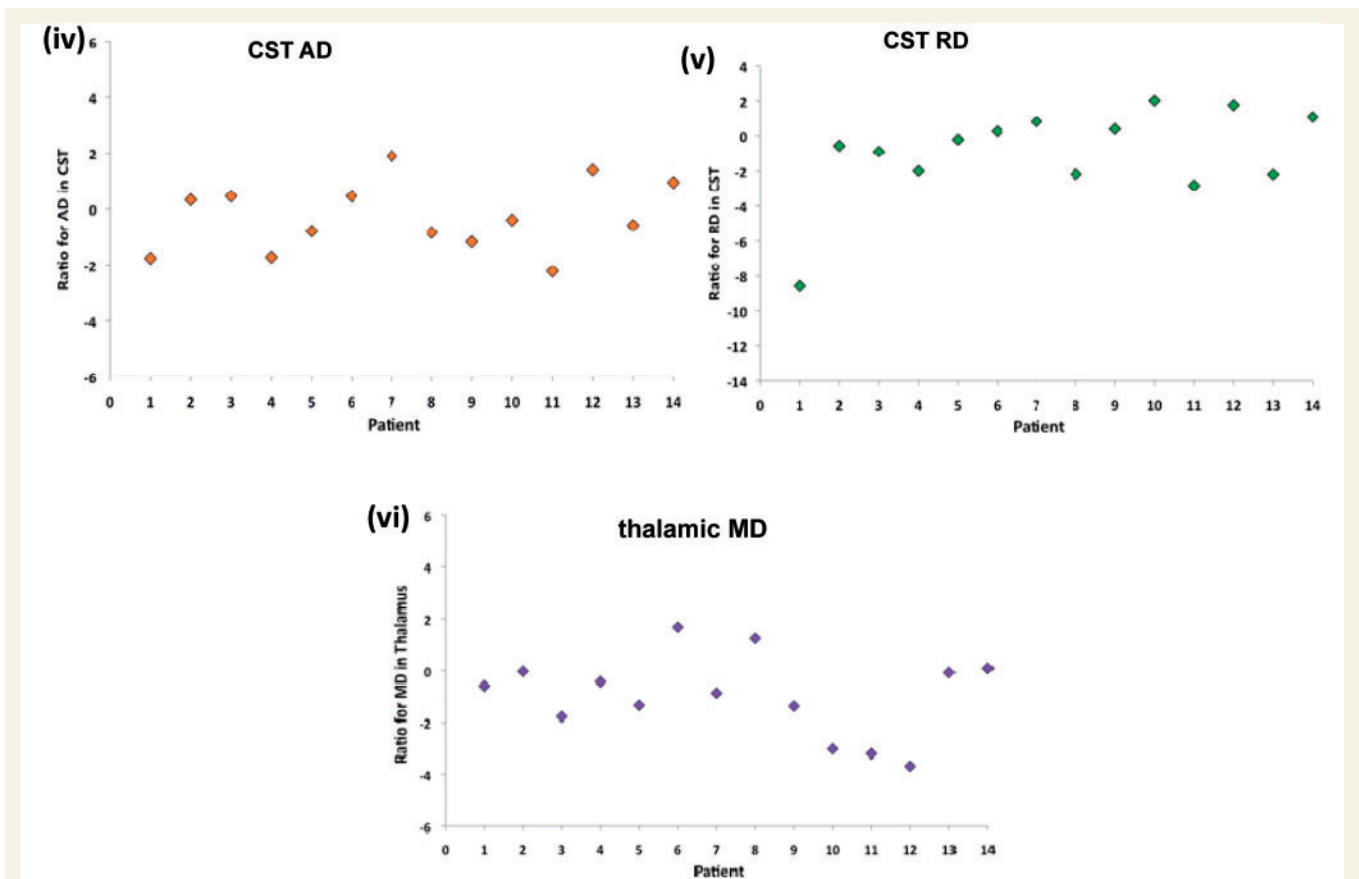


Figure 5 Continued.

the individual measurements, are shown in Fig. 5Bii–vi. Consistent with imaging results, the mean increase in EDSS score tended to be greater in the pretreatment compared with the treatment phase (Table 3).

## Discussion

This study suggests that blocking ASIC1 channels, which play a role in the development of irreversible tissue damage, may exert neuroprotective effects in patients with progressive multiple sclerosis.

Extending our previous findings (Vergo *et al.*, 2011), we demonstrated that in chronic progressive multiple sclerosis brains, there is an increased ASIC expression even within inactive lesions. This observation provides further evidence that ASIC1 contributes to neuroaxonal damage and does so even in the absence of acute inflammation. In combination with previous results demonstrating beneficial effects of amiloride in chronic relapsing experimental autoimmune encephalomyelitis, these results formed the premise to support our translational study testing the neuroprotective effect of amiloride in progressive patients.

We therefore recruited a group of patients with primary progressive multiple sclerosis participating in a longitudinal imaging protocol to assess whether amiloride could impact on surrogate imaging markers of neurodegeneration. This study compared

their rates of brain atrophy and tissue damage during the pretreatment and the amiloride treatment phase. In Alzheimers disease, this model of run-in design to measure the effect of treatment on each individual's rate of atrophy, and the use of multiple sampling, is thought to increase power (Schott *et al.*, 2006; Frost *et al.*, 2008). By adopting this approach in combination with the large neuroprotective effect in animal models (Friese *et al.*, 2007; Vergo *et al.*, 2011), we were able to detect statistically significant MRI evidence of benefit during the amiloride phase in progressive multiple sclerosis using small numbers of patients.

The significant reduction in the rate of whole-brain atrophy during the treatment phase supports a neuroprotective effect of amiloride. Such registration-based methods for quantification of whole-brain atrophy are considered reproducible and sensitive markers of neurodegeneration in multiple sclerosis that are relevant for testing the neuroprotective effects of treatment strategies (Barkhof *et al.*, 2009). However, changes in whole-brain atrophy rate lack pathological specificity and are relatively indiscriminate in regard to effects of neuroaxonal and myelin loss on brain volume that may be offset by other pathophysiological processes in multiple sclerosis, such as gliosis. Thus, in parallel to a reduction in the rate of whole-brain atrophy, we also demonstrated significant changes in the combined diffusion tensor imaging measures during amiloride treatment compared with the pretreatment phase, suggesting less damage in remaining brain tissue and providing hypothetical mechanisms through which amiloride might be



exerting a protective effect. Both changes of axial diffusivity, thought to be sensitive to axonal damage, and radial diffusivity, thought to reflect myelin loss, in the white matter tracts contributed to the treatment effect. Although this premise may be rather simplistic, our observations would support a neuroprotective and myeloprotective effect of amiloride that paralleled our current and previous findings in experimental studies (Friese *et al.*, 2007; Vergo *et al.*, 2011). The regions of interest for diffusion tensor imaging analysis included the corpus callosum and corticospinal tract (white matter) and thalamus (grey matter). The corpus callosum is the largest compact white matter fibre bundle of the human brain involved in interhemispheric transfer. Early corpus callosum damage can predict the progression of disability in patients with primary progressive multiple sclerosis over the long term (Bodini *et al.*, 2012). The corticospinal tract is the main motor white matter tract, in which pathology correlates with clinical disability (Reich *et al.*, 2008). The thalamus is also implicated in long-term accumulation of disability in patients with primary progressive multiple sclerosis (Houtchens *et al.*, 2007; Rocca *et al.*, 2010). Previous pathological and imaging studies have demonstrated marked neurodegeneration in the thalamus in multiple sclerosis (Cifelli *et al.*, 2002), with measures of volume loss paralleling measures of altered tissue integrity, suggesting that the thalamus is a suitable structure to measure the effects of neuroprotective treatment. Our results support the use of both atrophy and tissue integrity measures in assessing neuroprotection.

Although not powered to measure changes in clinical disability, our study showed that, consistent with the imaging outcomes, the mean change in EDSS score tended to be greater in the pretreatment compared with the treatment phase, encouraging further studies powered to detect a clinical effect of the drug.

We recognize the potential limitations of this study. Regression towards the mean often leads to a reduction in disease activity after recruitment in a clinical trial. However, because recruitment began at the beginning of the observational phase, regression towards the mean should have predominantly occurred at onset of the pretreatment phase, but this may have had some effect throughout. Although we cannot rule out an effect of the scanner upgrade on measurements, there is no reason for there to be a systematic reduction in rate of change. Additionally, brain volume measurements are reproducible even across different magnetic resonance machines of similar magnetization strength, and brain atrophy is used as an outcome in multi-centre studies across sites (Gasparini *et al.*, 2001). Whilst the study was open labelled, the imaging measures were assessed blindly and in random order, and therefore, the primary study outcomes were not subject to an unblinding bias. The small sample size and the single-site study may raise questions with regard to the robustness of the data. However, the purpose of this study was to obtain preliminary data on the potential neuroprotective effects of amiloride in patients with multiple sclerosis.

Our results extend the evidence that acid-sensing ion channels play a role in neurodegeneration within patients with chronic progressive multiple sclerosis and support a neuroprotective effect with amiloride, which blocks these channels. An additional advantage being that amiloride is a clinically licensed and safe diuretic with an extensive track record of human use and thus offers a

potentially rapid and inexpensive translation to patients. These results support the need for larger randomized placebo controlled studies to measure the neurodegenerative outcomes of amiloride both in the acute inflammatory setting and in progressive disease. However, beyond the progressive forms of multiple sclerosis because amiloride can reduce neuronal and myelin loss in experimental models by acting downstream of inflammation (Friese *et al.*, 2007; Vergo *et al.*, 2011), this neuroprotective approach may work in conjunction with immunomodulatory drugs also in the relapsing form of the disease. Our findings also support the use of imaging markers of neurodegeneration such as brain atrophy and tissue integrity measures in the development of neuroprotective strategies in multiple sclerosis (Barkhof *et al.*, 2009), as well as in other neurodegenerative conditions of the CNS.

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