

# Casting light on multiple sclerosis heterogeneity: the role of *HLA-DRB1* on spinal cord pathology

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Clinical heterogeneity in multiple sclerosis is the rule. Evidence suggests that *HLA-DRB1\*15* may play a role in clinical outcome. Spinal cord pathology is common and contributes significantly to disability in the disease. The influence of *HLA-DRB1\*15* on multiple sclerosis spinal cord pathology is unknown. A post-mortem cohort of pathologically confirmed cases with multiple sclerosis ( $n = 108$ , 34 males) with fresh frozen material available for genetic analyses and fixed material for pathology was used. *HLA-DRB1* alleles were genotyped to select a subset of age- and sex-matched *HLA-DRB1\*15*-positive ( $n = 21$ ) and negative ( $n = 26$ ) cases for detailed pathological analyses. For each case, transverse sections from three spinal cord levels (cervical, thoracic and lumbar) were stained for myelin, axons and inflammation. The influence of *HLA-DRB1\*15* on pathological outcome measures was evaluated. Carriage of *HLA-DRB1\*15* significantly increased the extent of demyelination (global measure  $15+ : 23.7\%$  versus  $15- : 12.16\%$ ,  $P = 0.004$ ), parenchymal (cervical,  $P < 0.01$ ; thoracic,  $P < 0.05$ ; lumbar,  $P < 0.01$ ) and lesional inflammation (border,  $P = 0.001$ ; periplaque white matter,  $P < 0.05$ ) in the multiple sclerosis spinal cord. *HLA-DRB1\*15* influenced demyelination through controlling the extent of parenchymal inflammation. Meningeal inflammation correlated significantly with small fibre axonal loss in the lumbar spinal cord ( $r = -0.832$ ,  $P = 0.003$ ) only in *HLA-DRB1\*15*-positive cases. *HLA-DRB1\*15* significantly influences pathology in the multiple sclerosis spinal cord. This study casts light on the role of *HLA-DRB1\*15* in disease outcome and highlights the powerful approach of using microscopic pathology to clarify the way in which genes and clinical phenotypes of neurological diseases are linked.

**Keywords:** multiple sclerosis; spinal cord; genetics; pathology; outcome

## Introduction

Multiple sclerosis is a common CNS disease characterized by discrete foci of inflammatory demyelination and axonal loss. Clinical heterogeneity is the rule with the greatest determinant of disability being entry into the progressive phase, which in most patients, is characterized by a relentless myelopathy

(Kremenchutzky *et al.*, 2006). Pathological studies demonstrate that axonal loss in functionally relevant tracts primarily located in the spinal cord, such as the corticospinal and sensory tracts, is the likely substrate for this irreversible neurological disability (Wujek *et al.*, 2002; DeLuca *et al.*, 2004; Tallantyre *et al.*, 2010). The cause of axonal loss in multiple sclerosis is unknown.

Recent genome wide association studies have identified multiple sclerosis susceptibility genes mostly related to inflammatory pathways, with the human leukocyte antigen Class II region (namely, the extended *HLA-DRB1\*1501*-bearing haplotype) having the strongest effect (Sawcer *et al.*, 2011). These genetic findings fuel support for the concept that multiple sclerosis is principally a chronic inflammatory disease with the T cell playing a central role.

Several lines of evidence suggest that multiple sclerosis susceptibility genes, particularly *HLA-DRB1\*15*, also influence the phenotypic expression of the disease although some of the evidence on this aspect of the disease is conflicting (Masterman *et al.*, 2000; Barcellos *et al.*, 2002; DeLuca *et al.*, 2007; Ramagopalan *et al.*, 2008; Okuda *et al.*, 2009; Sombekke *et al.*, 2009). Underlying genetic differences probably contribute not only to clinical heterogeneity observed between patients with multiple sclerosis, but also to the pathological differences between them. It remains unclear, however, how and to what extent *HLA-DRB1\*15* influences important pathological processes only visible at the microscopic level.

In this study, we provide new evidence about the influence of the main multiple sclerosis susceptibility allele, *HLA-DRB1\*15*, on the extent of demyelination, inflammation, and axonal loss in clinically eloquent tracts in the multiple sclerosis spinal cord.

## Materials and methods

### Study population

A human autopsy cohort of pathologically confirmed patients with multiple sclerosis ( $n = 108$ ) derived from UK Multiple Sclerosis Tissue Bank at Imperial College, London, with DNA available for genetic analyses and fixed material available for pathological study, and age- and sex-matched control ( $n = 10$ ) fixed material from the Thomas Willis Oxford Brain Collection, Oxford University Hospitals NHS trust, was used with appropriate consent as per Human Tissue Authority (HTA) guidelines and relevant ethics committee approval.

### Neuropathological evaluation

Adjacent formalin-fixed, paraffin-embedded transverse sections taken from three levels along the length of the spinal cord (cervical, thoracic and lumbar levels) were immunostained with primary antibodies to demonstrate myelin and inflammation, and impregnated with Palmgren's silver to demonstrate axons (Supplementary Table 1). Total and notional tract (i.e. dorsal column, lateral corticospinal tract and anterior corticospinal tract) cross-sectional spinal cord areas, axonal density, total axonal number estimates and total proportional plaque load areas (where applicable) were obtained as outlined in Fig. 1 and Supplementary Fig. 1 (DeLuca *et al.*, 2004, 2006). Stage of demyelination (i.e. acute, border active or chronic inactive) was determined using established criteria based on the intensity and distribution of microglial infiltrate in demyelinated regions (Diaz-Sanchez *et al.*, 2006). Quantitative scoring of inflammation in the spinal cord parenchyma, meninges, and plaques is described in Fig. 1.

### Genetics

DNA extraction from fresh frozen cerebellar tissue and *HLA-DRB1* high resolution genotyping were performed on all cases with multiple

sclerosis using established methods. Genotype scores were confirmed by two independent observers.

## Statistical analysis

Linear regression models were fitted to evaluate the influence of disease or *HLA-DRB1\*15* status on continuous pathological outcome measures while controlling for age, sex and duration of disease, as appropriate. Partial correlations were controlled for age, sex, and duration of disease. Model assumptions were evaluated analytically and graphically and variables were transformed, as appropriate. Data are expressed as mean  $\pm$  standard error. All tests of hypotheses were two-sided and conducted at significance level 0.05. Analyses were carried out using SPSS v.20 software.

## Results

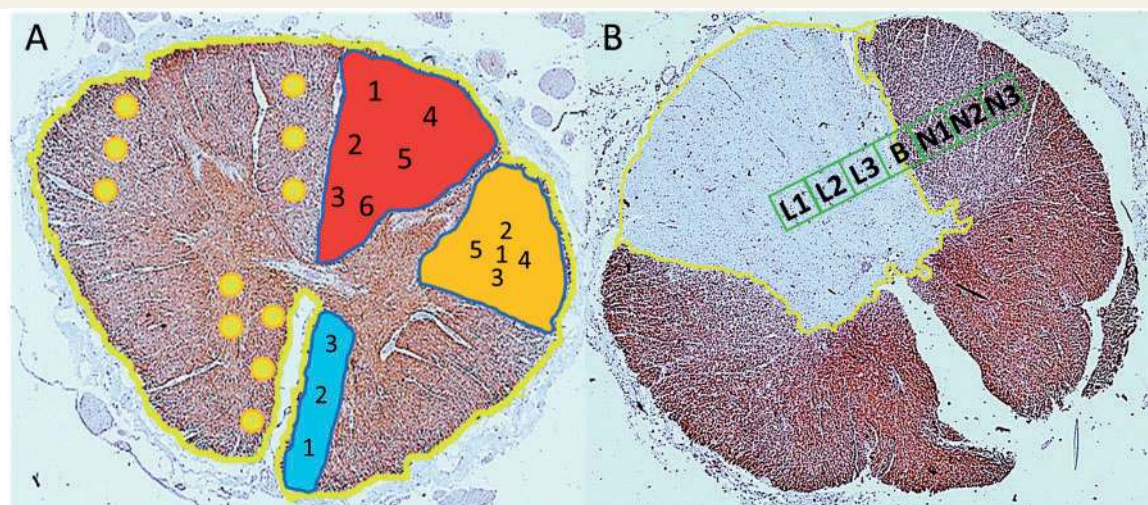
### Genetics

DNA extraction and *HLA-DRB1* typing were successful in 104 patients from which a subset of age- and sex-matched *HLA-DRB1\*15*-positive ( $n = 21$ ) and negative ( $n = 26$ ) cases with multiple sclerosis were selected for detailed pathological analyses. Duration of disease, disease course and post-mortem interval did not differ significantly between patient groups. Clinical details are given in Supplementary Table 2.

## Neuropathological findings: multiple sclerosis versus controls

### Demyelination

Demyelinating lesions were found in 37/47 (78%) of cases with multiple sclerosis and involved 83/141 (58%) of the spinal cord levels studied. In patients with lesions, a global mean of 22.7% of the total cord area was demyelinated. The extent of demyelination varied by spinal cord level being significantly greater in the upper compared to lower spinal cord (cervical = 20.9%; thoracic = 18.4%; lumbar = 13.3%; upper (cervical and thoracic) versus lumbar cord  $P = 0.007$  by Wilcoxon Signed Ranks Test; (Supplementary Fig. 2). Of 155 plaques analysed in detail, 16.7% were acute, 42.6% were border active, and 40.6% were chronic inactive. The proportion of active lesions (i.e. acute and border active) was similar throughout the length of cord (cervical, 66.7% versus lumbar, 63.1%,  $P = 0.614$ ). Significantly more CD3+ T cells were found within the lesional centre and border in acute plaques compared to border active ( $P < 0.001$ ) and chronic plaques ( $P < 0.001$ ). Periplaque white matter regions did not show significant differences in T cell infiltration between lesion subtypes (Supplementary Fig. 2). Spinal cord level influenced the extent of plaque inflammation; relative to the lumbar cord, active plaques at the cervical level had significantly more CD3+ T cells at their borders (cervical,  $133 \pm 9$  cells/mm<sup>2</sup> versus lumbar,  $82 \pm 13$  cells/mm<sup>2</sup>,  $P < 0.003$ ) and in periplaque white matter



**Figure 1** Quantitative spinal cord measures. (A and B) Axial view of multiple sclerosis thoracic cord stained for myelin (PLP). (A) Total spinal cord area measures were undertaken by outlining the perimeter of the cord (illustrated as yellow line). Cross-sectional areas of specific tracts were measured in a standardized way as follows: (i) dorsal columns (red) were bordered posteriorly by the circumferential edge of the spinal cord, medially by central median fissure, and laterally by dorsal horn; (ii) lateral corticospinal tracts (orange) were bordered anteriorly by a horizontal line extending laterally from the most posterior part of the grey matter commissure out to the lateral border of the cord, laterally by the circumferential edge of the spinal cord, and medially by the dorsal horn; and (iii) anterior corticospinal tracts (blue) were bordered posteriorly by the most anterior part of grey matter commissure, laterally by a horizontal line extending from medial edge of anterior horn out to the anterior border of the cord, medially by edge of anterior fissure. Axonal measures were obtained at  $\times 400$  magnification for each tract and on each side for each level investigated with dorsal column tract axons sampled in six locations, lateral corticospinal tract axons sampled in five locations, and anterior corticospinal tract axons sampled in three locations in the orientation shown. Measures of inflammation in the normal appearing white matter and normal appearing grey matter were obtained at  $\times 200$  magnification by manually counting positively labelled cells (CD3+, CD8+, CD20+) in the three tracts and anterior horn on each side distant from the border of adjacent plaques in the orientation shown (yellow circles; values reported per  $\text{mm}^2$ ). Measures of microglia/macrophage infiltration (PGM1) were obtained in the same regions as for normal appearing white matter/normal appearing grey matter inflammation. Measures of meningeal inflammation were obtained at  $\times 400$  magnification by manually counting positively labelled cells (CD3+, CD8+, CD20+) subjacent to arachnoid/pial surfaces along the entire perimeter of the cord with overlying meninges (values reported per mm). (B) Estimates of plaque load area and inflammation. Plaque load area measures were obtained by outlining borders of clear myelin loss using PLP-stained sections (outlined in yellow). Plaque load inflammation was quantified as follows: manual counts of CD3+ and CD8+ cells at  $\times 400$  magnification were obtained in 284 strips of seven sequential fields extending from the lesion centre (L1, L2, L3) to the border (B) and periplaque white matter (N1, N2, N3). Estimates of lesional inflammation (mean of L1–L3), border (B) and periplaque white matter inflammation (mean of N1–N3) were obtained (values reported per  $\text{mm}^2$ ).

(cervical,  $63 \pm 48$  cells/ $\text{mm}^2$  versus lumbar,  $49 \pm 6$  cells/ $\text{mm}^2$ ,  $P = 0.05$ ). Control cases showed no demyelination.

## Parenchymal inflammation

Parenchymal CD3+ T cell infiltration was diffuse and significantly increased in both normal appearing white and grey matter of cases with multiple sclerosis compared with control cases, with the majority of T cells being CD8+. In cases with multiple sclerosis, T-lymphocyte infiltration was more pronounced in normal appearing white matter compared with normal appearing grey matter, and in the cervical and thoracic cords compared to the lumbar cord (Supplementary Fig. 3). Normal appearing white matter CD3+ T cell infiltration correlated significantly between spinal cord levels (cervical versus thoracic:  $r = 0.819$ ,  $P < 0.001$ ; cervical versus lumbar:  $r = 0.898$ ,  $P < 0.001$ ; thoracic versus lumbar:  $r = 0.793$ ,  $P < 0.001$ ). CD3+ T cells were only rarely seen in controls. CD20+ B cells were occasionally detected throughout the multiple sclerosis spinal cord but were virtually absent in controls.

## Meningeal inflammation

Meningeal T cell inflammation was significantly increased in cases with multiple sclerosis compared with controls at the cervical and thoracic cord levels, with CD8+ cells constituting the majority of CD3+ T cells. In comparison, meningeal B cells were significantly fewer in cases with multiple sclerosis at each spinal cord level and were rarely detected in controls (Supplementary Fig. 3).

## Area, axonal density and total axonal numbers

Multiple sclerosis cases showed statistically significant reductions in total cord and notional tract areas at all levels compared to controls, with the upper spinal cord being most severely affected (Supplementary Fig. 4). Axonal density and total axonal numbers were significantly reduced throughout the length of the spinal cord, with small fibres being preferentially lost (Supplementary Table 3).

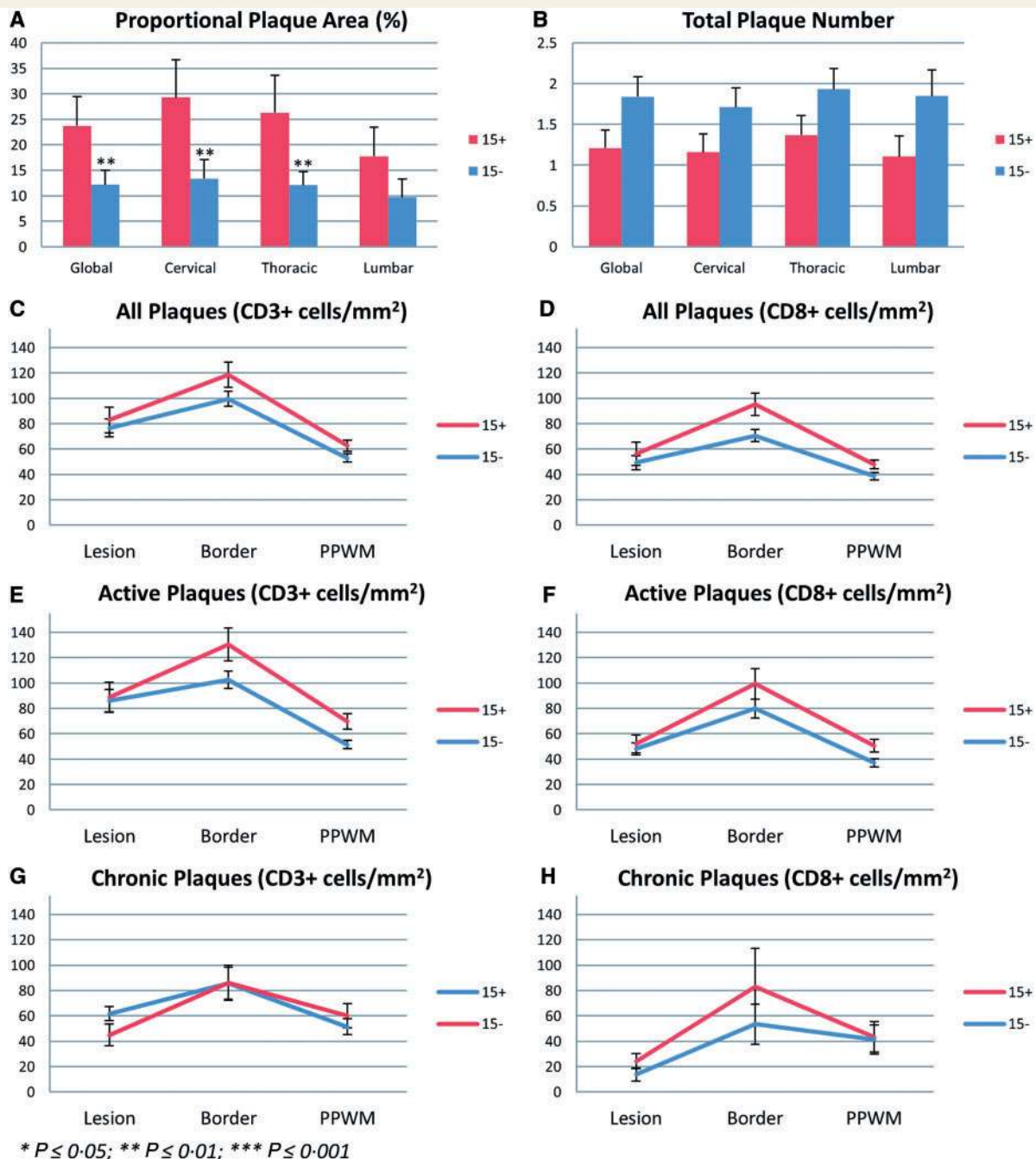


# Genetic–pathology relationships in multiple sclerosis

## Demyelination

*HLA-DRB1\*15*-positive patients had significantly greater plaque load burden globally (23.7% versus 12.2%,  $P = 0.004$ ) and at

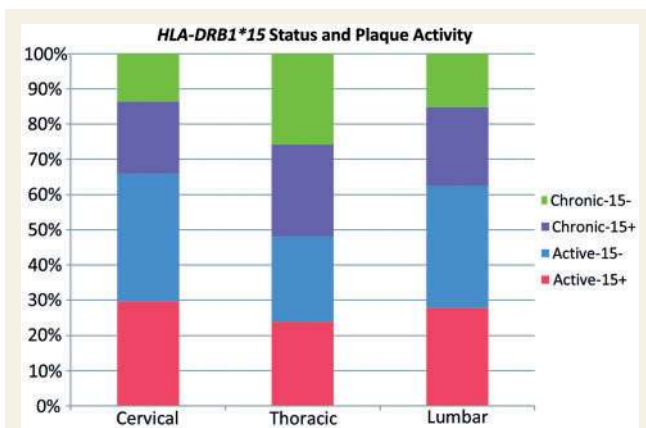
cervical (29.3% versus 13.4%,  $P = 0.003$ ), thoracic (26.3% versus 12.1%,  $P = 0.008$ ) and lumbar (17.7% versus 9.69%,  $P = 0.074$ ) levels. *HLA-DRB1\*15* status did not influence the number of plaques at each level investigated, except for at the cervical level where plaques were less numerous ( $15+$ ,  $1.16 \pm 0.22$  versus  $15-$ ,  $1.71 \pm 0.24$ ,  $P = 0.08$ ) but typically larger (Fig. 2). Cases with multiple sclerosis without evidence of demyelination ( $n = 10$ ) were distributed similarly between *HLA-DRB1\*15*-positive ( $n = 6$ ) and *HLA-DRB1\*15*-negative ( $n = 4$ ) cases.



**Figure 2** *HLA-DRB1\*15* status and demyelination. Bar graphs of proportional plaque load (A) and plaque number (B) in *HLA-DRB1\*15* positive and negative cases with multiple sclerosis both globally and at each spinal cord level studied. Line graphs of the extent of CD3+ (C, E and G) and CD8+ (D, F and H) T cell inflammation in active and chronic plaques, and all plaques combined.

Stage of plaque demyelinating activity did not vary significantly between *HLA-DRB1\*15*-positive and negative groups (Fig. 3). Active plaques in *HLA-DRB1\*15*-positive cases had significantly greater CD3+ and CD8+ T cell infiltration at their borders (CD3+,  $124.4 \pm 12.9$  cells/mm<sup>2</sup> versus  $100.5 \pm 6.7$  cells/mm<sup>2</sup>,

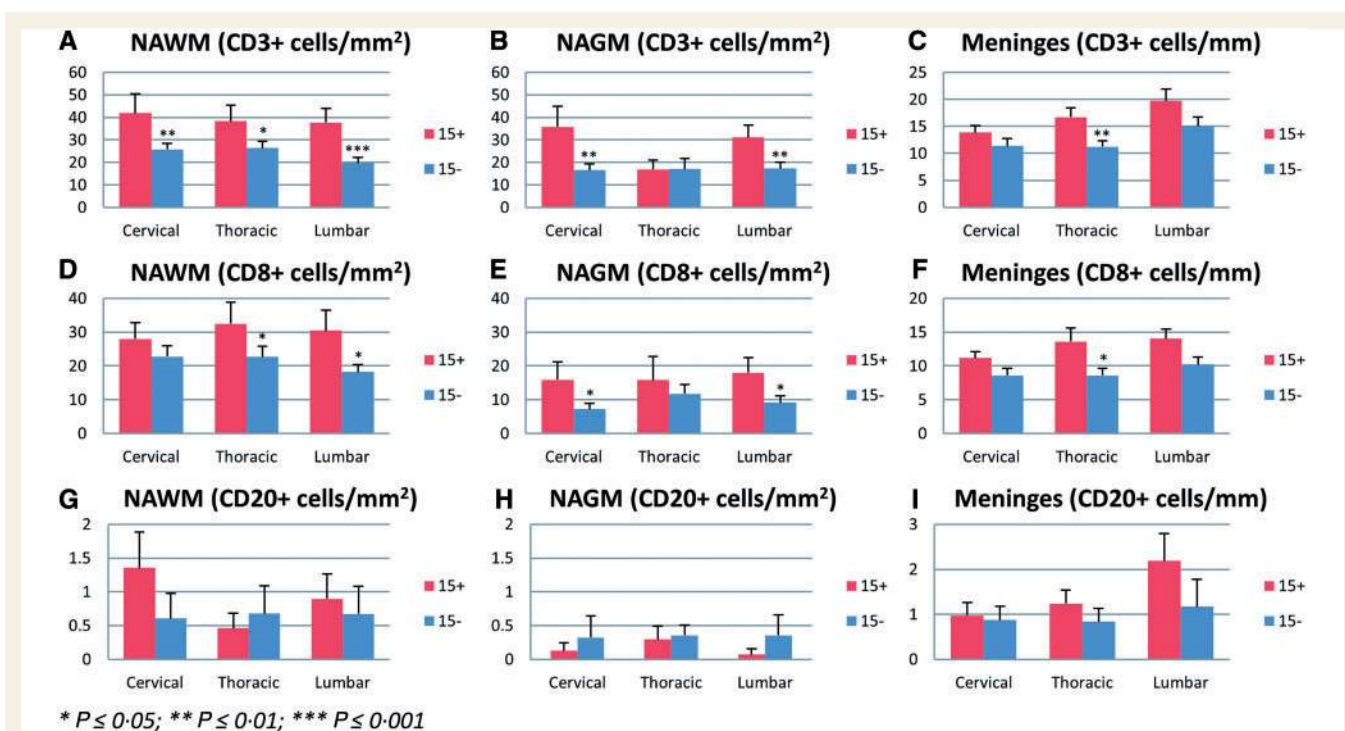
$P = 0.01$ ; CD8+,  $108.2 \pm 29.4$  cells/mm<sup>2</sup> versus  $78.5 \pm 5.3$  cells/mm<sup>2</sup>,  $P = 0.001$ ) and periplaque white matter (CD3+,  $69.4 \pm 6.2$  cells/mm<sup>2</sup> versus  $51.5 \pm 3.2$  cells/mm<sup>2</sup>,  $P = 0.02$ ; CD8+,  $49.7 \pm 3.8$  cells/mm<sup>2</sup> versus  $42.1 \pm 3.2$  cells/mm<sup>2</sup>,  $P = 0.023$ ) (Fig. 2). *HLA-DRB1\*15* status did not influence the extent of CD3+ T cell inflammation in chronic plaques (Fig. 2).



**Figure 3** Influence of *HLA-DRB1\*15* status on plaque activity. Histogram of proportion of active (i.e. acute and border active) and chronic inactive plaques in *HLA-DRB1\*15*-positive and -negative cases divided by spinal cord level. Note that plaque activity was defined by the extent and distribution of PGM1-staining as previously described (Diaz-Sanchez *et al.*, 2006).

## Parenchymal inflammation

Multiple sclerosis cases bearing the *HLA-DRB1\*15* allele demonstrated significantly more CD3+ T cell inflammation in both normal appearing white and grey matter when controlling for presence and activity of plaques at the respective spinal cord levels (normal appearing white matter: cervical, 15+:  $41.9 \pm 8.1$  cells/mm<sup>2</sup> versus 15–:  $25.7 \pm 2.9$  cells/mm<sup>2</sup>,  $P < 0.01$ ; thoracic, 15+:  $38.3 \pm 6.9$  cells/mm<sup>2</sup> versus 15–:  $26.3 \pm 6.9$  cells/mm<sup>2</sup>,  $P < 0.05$ ; lumbar, 15+:  $37.7 \pm 6.2$  cells/mm<sup>2</sup> versus 15–:  $19.6 \pm 2.6$  cells/mm<sup>2</sup>,  $P < 0.01$ ; normal appearing grey matter: cervical, 15+:  $35.7 \pm 9.4$  cells/mm<sup>2</sup> versus 15–:  $16.5 \pm 2.8$  cells/mm<sup>2</sup>,  $P < 0.01$ ; thoracic, 15+:  $16.8 \pm 4.2$  cells/mm<sup>2</sup> versus 15–:  $16.9 \pm 4.7$  cells/mm<sup>2</sup>, n.s.; lumbar, 15+:  $31.1 \pm 5.3$  cells/mm<sup>2</sup> versus 15–:  $17.2 \pm 2.9$  cells/mm<sup>2</sup>,  $P < 0.01$ ). Similar findings were found for CD8+ T cells in both normal appearing white and grey matter (Fig. 4). *HLA-DRB1\*15* status did not impact the extent of CD20+ B cell inflammation (Fig. 4) or microglial/macrophage (PGM1) inflammation (Supplementary Table 4) in normal appearing white or grey matter at any spinal cord level studied.



**Figure 4** *HLA-DRB1\*15* status and inflammation. Bar graphs of the extent of inflammation (CD3+, CD8+, CD20+) in normal appearing white matter (A, D and G), normal appearing grey matter (B, E and H), and meninges (C, F and I) in *HLA-DRB1\*15*-positive and -negative cases with multiple sclerosis.

## Meningeal inflammation

*HLA-DRB1\*15* did not have a statistically significant influence on the extent of CD3+ and CD8+ T lymphocyte meningeal inflammation at any spinal cord level except at the thoracic cord (CD3+: 15+,  $16.71 \pm 1.71$  cells/mm<sup>2</sup> versus 15–,  $11.22 \pm 1.13$  cells/mm<sup>2</sup>,  $P = 0.01$ ; CD8+: 15+,  $13.65 \pm 8.58$  cells/mm<sup>2</sup> versus 15–,  $8.58 \pm 1.01$  cells/mm<sup>2</sup>,  $P = 0.03$ ) (Fig. 4). *HLA-DRB1\*15* status did not impact the extent of CD20+ B cell inflammation in the meninges at any spinal cord level studied (Fig. 4).

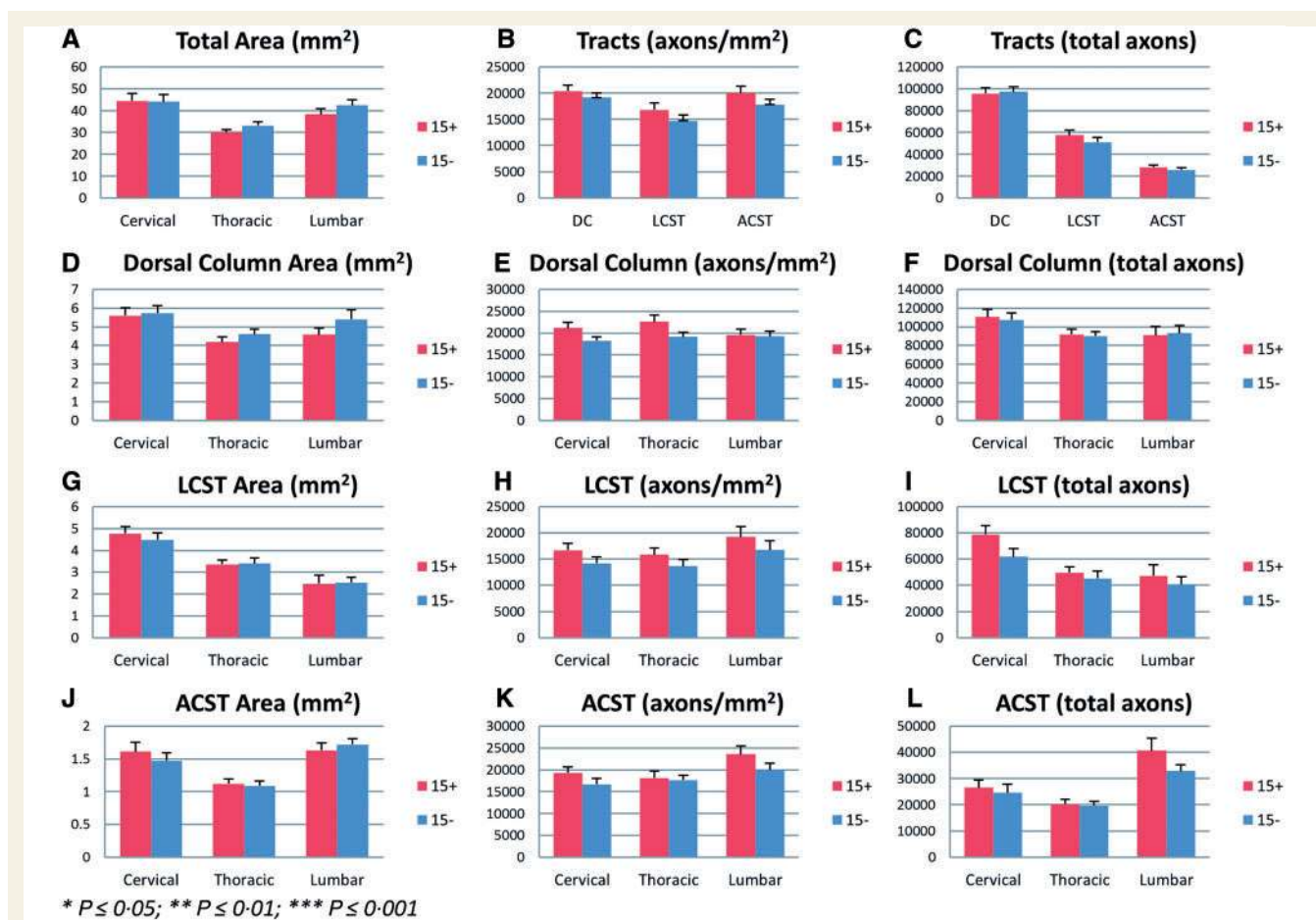
## Area, axonal density and total axonal numbers

Measures of total spinal cord and notional tract cross-sectional areas did not differ significantly between *HLA-DRB1\*15* groups at each spinal cord level (Fig. 5). Similarly, *HLA-DRB1\*15* status did not influence axonal density and total axonal number estimates in the tracts examined (Fig. 5).

## Relationships between demyelination, inflammation and axonal loss and the influence of *HLA-DRB1\*15*

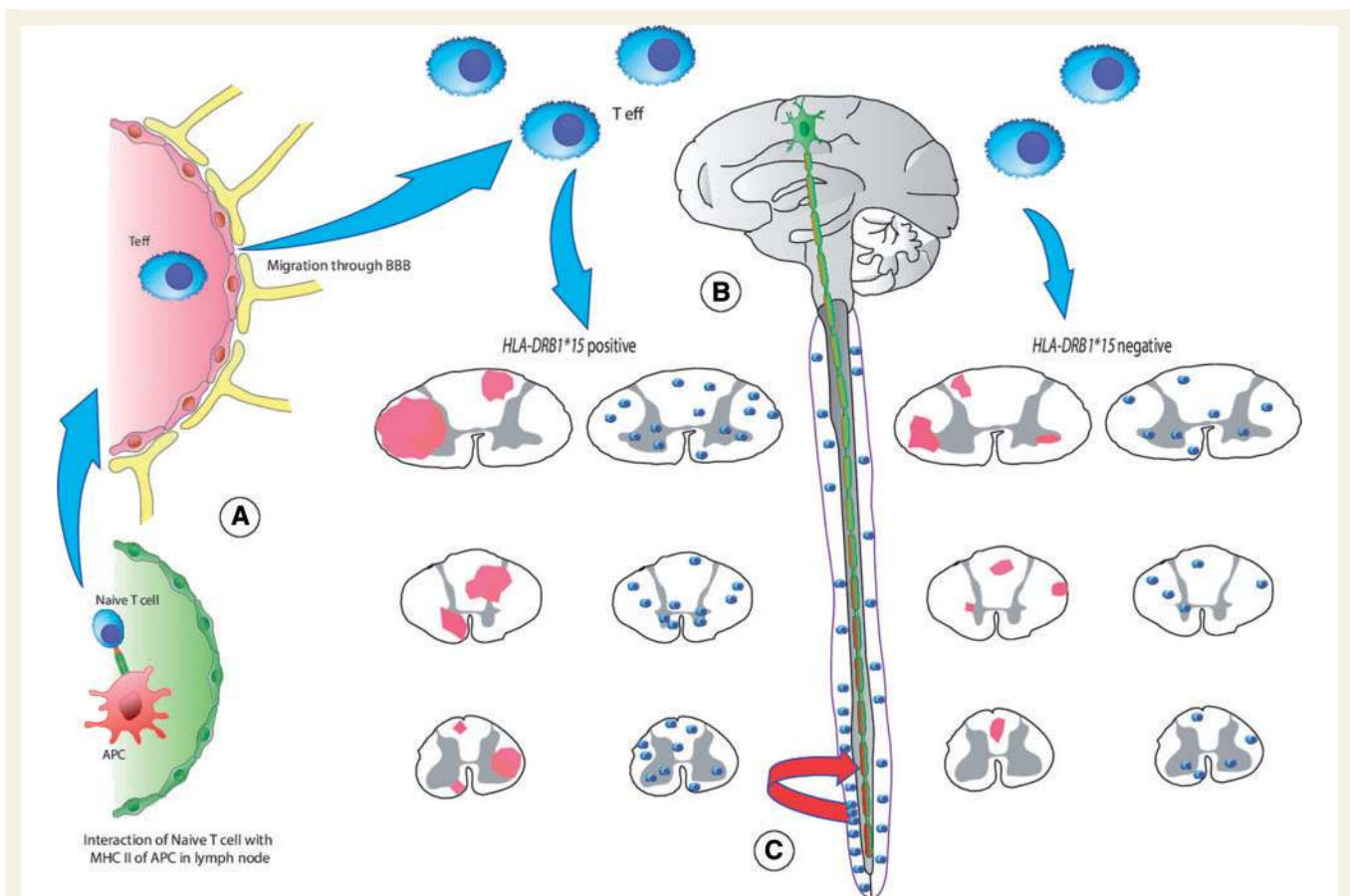
On segregation by *HLA-DRB1\*15* status, significant correlations between demyelination, inflammation and axonal loss were restricted to cases bearing the *HLA-DRB1\*15* allele. On controlling for the extent of demyelination, *HLA-DRB1\*15* had a significant impact on parenchymal inflammation ( $P = 0.006$ ). Conversely, when parenchymal inflammation was controlled, *HLA-DRB1\*15* had no influence on the extent of demyelination ( $P = 0.904$ ). These findings suggest that *HLA-DRB1\*15* exerts its influence on demyelination primarily through controlling the extent of parenchymal inflammation.

The relationship between inflammation and axonal loss in *HLA-DRB1\*15*-positive cases was complex. In *HLA-DRB1\*15*-positive cases, parenchymal inflammation correlated significantly with meningeal inflammation in the lumbar spinal cord ( $r = 0.503$ ,  $P < 0.05$ , Spearman rank coefficient), but not with



**Figure 5** *HLA-DRB1\*15* status and area/axonal measures. Bar graphs of total cord area (A), tract-specific axon density (B), tract-specific total axon number (C), along with cross-sectional area, axon density, and total axon number of the dorsal column (D, E and F), lateral corticospinal tract (G, H and I), and anterior corticospinal tract (J, K and L) in *HLA-DRB1\*15*-positive and negative cases with multiple sclerosis.





**Figure 6** Influence of *HLA-DRB1\*15* on multiple sclerosis spinal cord pathology. (A) Schematic representation of antigen presentation in lymph node (green) and trafficking through the blood brain barrier of blood vessel (red with yellow astrocytic foot processes). Naïve T cells in the periphery are activated when they encounter Major Histocompatibility Complex class II restricted antigen presenting cells (APCs) that present cognate antigen. Activation results in generation of myelin specific effector T cells (T eff) that subsequently penetrate the blood brain and/or blood CSF barriers to enter the CNS. (B) Schematic representation of brain and spinal cord with cervical, thoracic and lumbar cord levels presented (top to bottom). The extent of demyelination (pink) and inflammation (blue T eff cells) are illustrated in *HLA-DRB1\*15*-positive (left) versus *HLA-DRB1\*15*-negative (right) patients. Patients positive for *HLA-DRB1\*15* allele have an increased T cell inflammatory response, larger plaques, and an increased extent of global demyelination in the spinal cord, particularly in the cervical region, compared with *HLA-DRB1\*15*-negative patients. (C) Schematic representation of spinal cord meninges (purple), meningeal T cells in the subarachnoid space (blue), and a small diameter corticospinal axonal process within the spinal cord (green). While *HLA-DRB1\*15* status does not directly influence meningeal inflammation or axonal loss, meningeal inflammation was significantly correlated with small fibre axonal loss in the lumbar spinal cord only in *HLA-DRB1\*15*-positive cases ( $r = -0.832$ ,  $P = 0.003$ ).

axonal loss. However, meningeal inflammation itself correlated significantly with small fibre axonal loss of the lumbar lateral corticospinal tract in these cases ( $r = -0.832$ ,  $P = 0.003$ ) (Fig. 6). This is in contrast to *HLA-DRB1\*15* negative cases where axonal loss did not correlate with measures of demyelination and inflammation (meningeal or parenchymal) at any level or in any tract.

## Discussion

We report, for the first time, that *HLA-DRB1\*15* influences significantly the extent of demyelination and inflammation assessed microscopically throughout the length of the spinal cord and indirectly contributes to tract-specific, length dependent and size-selective axonal loss. These observations are novel and

provide a critical link between a readily available *in vivo* genetic biomarker (*HLA-DRB1\*15* status) and the pathologic changes it mitigates which are only detectable, at present, after death.

In our cohort, demyelination in the multiple sclerosis spinal cord was common, affecting 78% of cases with multiple sclerosis, with the cervical cord being most severely affected and active plaques being most frequent (i.e. 59% were either acute or border active). Carriage of the *HLA-DRB1\*15* allele significantly increased the extent of demyelination (Sombekke *et al.*, 2009) through increased size of plaques, most notably in the upper cord, in accordance with some MRI studies (Qiu *et al.*, 2011). Interestingly, in contrast, the numbers of plaques did not differ significantly between *HLA-DRB1\*15*-positive and negative cases. When matched for stage of demyelinating activity, plaques from *HLA-DRB1\*15*-positive patients were significantly more

inflammatory suggesting that the Major Histocompatibility Complex region impacts the extent of demyelination through a heightened inflammatory response. The reason for the long recognized upper cord bias in demyelination is not clear. Regional differences in myelin protein composition (Trotter *et al.*, 1984), blood–brain barrier integrity (Loeffler *et al.*, 2011), vascular patterns (Fog, 1950) and susceptibility to trauma (Oppenheimer, 1978) have been proposed. However, direct experimental evidence is lacking.

The multiple sclerosis spinal cord was highly inflammatory even in areas distant from demyelination. Parenchymal inflammation was markedly increased compared with controls throughout the spinal cord, with the normal appearing white matter having significantly more T cell infiltration than normal appearing grey matter. Similar to the inflammation in plaques, *HLA-DRB1\*15* significantly increased the extent of T cell inflammation in both normal appearing white and grey matter when controlling for the presence and activity of plaques. Given that *HLA-DRB1\*15* status had no influence on the relative proportion and distribution of active versus chronic plaques, it is unlikely that the observed differences in parenchymal inflammation between allelotype groups is merely a consequence of nearby plaque activity. In fact, our results suggest that the opposite may be true. Models evaluating the relationship between *HLA-DRB1\*15* status, parenchymal T cell inflammation and global estimates of plaque load demonstrated that *HLA-DRB1\*15* exerts its main influence on increasing the extent of inflammation in normal appearing white matter, which in turn predisposes to increased demyelination. This suggests that patients with *HLA-DRB1\*15* have an increased baseline level of inflammation throughout the length of the spinal cord making them more susceptible to episodes of inflammatory demyelination, increasing their risk of clinically symptomatic disease. In support of this, *HLA-DRB1\*15*-positive patients have been shown to have an earlier age of onset (Masterman *et al.*, 2000; Barcellos *et al.*, 2002), more severe cognitive (Okuda *et al.*, 2009) and motor (DeLuca *et al.*, 2007) impairments, and an increased number of brain (Okuda *et al.*, 2009) and spinal cord (Sombekke *et al.*, 2009) lesions. Further, the presence of spinal cord lesions in patients with radiologically isolated syndrome increases the likelihood of subsequent development of a clinically isolated syndrome or progressive disease independent of brain lesions on MRI (Okuda *et al.*, 2011).

Meningeal inflammation was pronounced throughout the length of the spinal cord in our multiple sclerosis cohort. This finding extends those described by Androdias *et al.* (2010) where meningeal inflammation was described in the cervical spinal cord. Meningeal inflammation was not clearly linked to *HLA-DRB1\*15* status despite the finding of severe normal appearing white matter inflammation only in *HLA-DRB1\*15*-positive cases. The *HLA-DRB1\*15* allele can serve as a restriction element for myelin basic protein (MBP)-specific CD4 T cells that facilitates the binding of DR  $\beta$ -chains to the immunodominant MBP peptide (83–99) postulated to drive the immune response in multiple sclerosis (Pette *et al.*, 1990; Wucherpfennig *et al.*, 1994). Extracellular myelin can be detected in the meninges of patients with multiple sclerosis (Kooi *et al.*, 2009), and meningeal T cells associate with Major Histocompatibility Complex class II macrophages (Androdias

*et al.*, 2010) and secrete proinflammatory cytokines, which in an animal model have been shown to act in a paracrine fashion to mitigate inflammation in spinal cord normal appearing white matter (Soulika *et al.*, 2009). These observations support the concept that spinal cord meninges may form an immunological niche that, once established, dissociates partly from genetic control as evidenced in this study.

It is interesting that in this study *HLA-DRB1\*15*-positive status is associated with the number of CD8+ T cells that, while not directly influenced by *HLA-DRB1\*15*, may have been affected by cytokines secreted by CD4+ T cells. The finding that CD3+ T cell infiltration was more strikingly related to *HLA-DRB1\*15* status than CD8+ T cell infiltration suggests that CD4+ T cells may have had an influence. It would have been of interest to search for a relationship between *HLA-DRB1\*15* status and CD4+ T cell counts but technical limitations made this not possible.

In line with previous reports, we found that axonal loss in functionally important motor and sensory tracts in the multiple sclerosis spinal cord was extensive, wide-spread, and size-selective (DeLuca *et al.*, 2004; Tallantyre *et al.*, 2010). *HLA-DRB1\*15* did not influence directly the extent of axonal loss in any of the tracts studied.

Interaction between genetic and pathologic variables is shown here to be complex. On one hand, *HLA-DRB1\*15* most strongly influences parenchymal and lesional inflammation throughout the spinal cord, which in turn, may drive the size and overall extent of demyelination. On the other hand, parenchymal inflammation (but not *HLA-DRB1\*15*) relates closely to meningeal inflammation in the lumbar spinal cord which, in turn, correlates significantly with size-selective axonal loss.

Further complexity is introduced in the specific tracts of the spinal cord. The distal ends of small axons in the lumbar lateral corticospinal tract showed selective vulnerability to axonal degeneration secondary to meningeal inflammation in *HLA-DRB1\*15* cases. This is in concordance with previous reports which have showed that persistent activation of spinal cord innate immunity, once focal inflammatory infiltrates clear, leads to a progressive, length-dependent degeneration of small corticospinal tract axons in animal models of the disease (Soulika *et al.*, 2009). Human post-mortem studies have lent evidence to the idea that multiple sclerosis begins as a focal inflammatory demyelinating disease that later develops into a diffuse inflammatory process (Kutzelnigg *et al.*, 2005). While the former associates with acute axonal injury and focal axonal swelling (Ferguson *et al.*, 1997; Nikic *et al.*, 2011), the latter has been shown to be a key driver of axonal degeneration (Kutzelnigg *et al.*, 2005; Soulika *et al.*, 2009; Androdias *et al.*, 2010; Choi *et al.*, 2012). *HLA-DRB1\*15* appears to adversely influence both phases.

It is not surprising that the distal ends of small lateral corticospinal tract axons are susceptible to degeneration. By virtue of their length, these axons have extraordinary metabolic demands and, therefore, are particularly vulnerable to the multiple concerted mechanisms thought to precipitate axonal injury in multiple sclerosis (Dutta and Trapp, 2011). The neurotoxic inflammatory milieu of the lumbar meninges in *HLA-DRB1\*15* cases may tip a delicate homeostatic balance towards degeneration of distal lateral corticospinal tract axons leading to the length-dependent motor



symptoms commonly encountered in the progressive phase of the disease.

Our findings have important clinical implications. The efficacy of current immunotherapies is judged by the extent to which they reduce clinical relapses and/or MRI lesions. However, the extent to which these treatments reduce underlying spinal cord parenchymal and meningeal inflammation is not known. The central role of meningeal inflammation on axonal loss, particularly in *HLA-DRB1\*15*-positive cases, may explain, in part, the underwhelming impact of current treatments on long term clinical outcomes. Clinical trials may benefit from taking into account *HLA-DRB1\*15* status in monitoring long-term responses to treatment given the differential effects demonstrated here. Our findings favour starting treatment early as once spinal cord meningeal inflammation is established, it may form a neurotoxic immunological niche that is relatively unresponsive to treatment.

In this study, we have demonstrated the power of microscopic pathology to cast light on the influence of a well established risk factor in multiple sclerosis. Tissue-based pathology provides a crucial means to clarify the way in which genes and clinical phenotypes of neurological diseases are linked. The same principal is also starting to emerge in evidence about other neurodegenerative disease in addition to multiple sclerosis (Bennett *et al.*, 2009).

In summary, we demonstrate that the genetic factor, *HLA-DRB1\*15*, contributes significantly to the landscape of multiple sclerosis spinal cord pathology. Given the crucial role of spinal cord pathology in clinical outcomes, future studies aimed at unravelling the mechanisms by which *HLA-DRB1\*15* sculpts multiple sclerosis spinal cord pathology will be necessary to identify therapeutic targets that impact long-term disability.

## Supplementary material

Supplementary material is available at *Brain* online.

## Acknowledgements

We would like to thank Katie Morrison for technical assistance. The authors acknowledge the support of the UK Multiple Sclerosis Brain Bank for supplying tissue samples and clinical information presented in this study.

## Funding

GCD is supported by the AANF/CMSC John F. Kurtzke Clinician-Scientist Development Award and a Goodger Scholarship (University of Oxford), and MME and GCD receive support from the NIHR Biomedical Research Centre, Oxford.

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