Increased HLA-DR expression and cortical demyelination in MS links with HLA-DR15

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Increased HLA-DR expression and cortical demyelination in MS links with HLA-DR15

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Abstract

Objective
To investigate molecular changes in multiple sclerosis (MS) normal-appearing cortical gray matter (NAGM).

Methods
We performed a whole-genome gene expression microarray analysis of human brain autopsy tissues from 64 MS NAGM samples and 42 control gray matter samples. We further examined our cases by HLA genotyping and performed immunohistochemical and immunofluorescent analysis of all human brain tissues.

Results
HLA-DRB1 is the transcript with highest expression in MS NAGM with a bimodal distribution among the examined cases. Genotyping revealed that every case with the MS-associated HLA-DR15 haplotype also shows high HLA-DRB1 expression and also of the tightly linked HLA-DRB5 allele. Quantitative immunohistochemical analysis confirmed the higher expression of HLA-DRB1 in HLA-DRB1*15:01 cases at the protein level. Analysis of gray matter lesion size revealed a significant increase of cortical lesion size in cases with high HLA-DRB1 expression.

Conclusions
Our data indicate that increased HLA-DRB1 and -DRB5 expression in the brain of patients with MS may be an important factor in how the HLA-DR15 haplotype contributes to MS pathomechanisms in the target organ.

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Go to Neurology.org/NN for full disclosures. Funding information is provided at the end of the article.

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Multiple sclerosis (MS), the most common inflammatory neurologic disease affecting young adults, is a chronic autoimmune demyelinating disease of the CNS. If untreated, MS leads to disability in a substantial proportion of patients. The etiology of MS includes a complex genetic trait and several environmental risk factors, which act in concert and contribute to the main pathomechanisms including autoimmune inflammation, de- and remyelination, axonal and neuronal loss, astroglia activation, and metabolic changes. The relative severity of these factors leads to the enormous heterogeneity of MS with respect to clinical signs, course, and response to treatment, but also pathologic composition of demyelinated lesions. The pathologic hallmark of MS is the formation of focal areas of myelin loss in the CNS. Besides the most commonly described white matter lesions, extensive gray matter lesions can be found in the MS cerebral cortex. In addition to the well-described demyelinated gray matter lesions also diffuse gray matter abnormalities in nonlesional normally myelinated areas have been described. At the molecular level, little is known about changes in normal-appearing cortical gray matter (NAGM) and gray matter lesions in MS. In the last years, several transcriptome studies of MS brain tissues have been performed, and a number of possible pathomechanisms could be identified such as mitochondrial dysfunction, metabolic changes in astrocytes, inflammation, and oxidative stress. A limitation of all these studies is the low number of tissue samples and cases and consequently the limited statistical power. The problem is further accentuated by the heterogeneity of MS, reflected by the variable clinical course, different clinical symptoms and imaging findings, and variability in pathology. As part of our published studies, we collected a large number of well-characterized human brain tissue samples from control and MS cases.

Here, we compared the expression pattern of MS NAGM with control gray matter (GM) to understand if there are alterations that may underlie or contribute to the formation of the widespread cortical lesions as an important aspect of MS pathology.

**Methods**

**Tissue selection and characterization**

MS and control tissue samples were provided by the UK MS Tissue Bank (UK Multicentre Research Ethics Committee, MREC/02/2/39), funded by the MS Society of Great Britain and Northern Ireland registered charity 207495, or obtained from the archives of the Institute of Neuropathology at the University Medical Centre Göttingen. Additional control samples were provided by the Pathology Department of the University Hospital Basel. All cases were routinely screened by a neuropathologist to confirm diagnosis of MS and to exclude other confounding pathologies. In total, 104 gray matter tissue blocks from 34 control cases and 101 NAGM tissue blocks from 51 MS cases were used for this study (table 1, further details in table e-1, links.lww.com/NXI/A173). Criteria of in- and exclusion are described in figure 1A. Tissues were characterized further by staining for neuronal nuclei (NeuN) (neurons), oligodendrocyte transcription factor 2 (oligodendrocytes), myelin oligodendrocyte glycoprotein (MOG) (myelin), and CD68 (microglia) (figure 1B). Cryostat sections (12 μm) from fresh-frozen tissue blocks were stained as described before. Antibodies and detailed protocols are described in table e-2, A and B (links.lww.com/NXI/A173).

**Ethical approvals**

Ethical approvals for all human tissues used were given by the UK Multicentre Research Ethics Committee, MREC/02/2/39 for the cases from London, by the Ethics Committee of the University Hospital Basel for all cases from Basel, and by the ethical review committee of the University Medical Center Göttingen (#19/09/10) for all cases from Göttingen.

**RNA isolation and quality assessment**

Total RNA from gray matter tissue was isolated using the Zymo ZR RNA Microprep Kit (Zymo Research, Irvine, CA) as described before. Degraded (RNA integrity index < 6) and/or contaminated (260/280 nm ratio < 1.8; 230/280 nm ratio < 1.8) samples were excluded from the study.

**Microarray analysis and statistical analysis**

From initially 151 NAGM and control gray matter samples, 35 were excluded due to the RNA integrity index being below the threshold of 6, 8 samples were excluded due to incongruence between the sex stated in the case reports and Y-chromosome linked gene expression, and 2 samples were excluded for being clear outliers in the principal component analysis. In total, 42 tissue samples from 14 control and 64 tissue samples from 25 MS cases were used for the gene expression analysis between NAGM and control GM (table 1 and table e-1, links.lww.com/NXI/A173). To minimize experimental bias, microarray experiments were performed together. All samples used for the gene expression study originated from the UK MS Tissue Bank. Gene expression profiling was performed using the Illumina complementary DNA–mediated annealing, selection, extension, and ligation assay according to the manufacturer’s protocol (Part No. 15018210, Revision history D, April 2012, Illumina, San Diego, CA).
Table 1 Patient data

| Cases  | Sex | Cause of death                                      | p.m. time (h) | Age (a) | Disease duration (a) | MS type | No. of brain tissue blocks | No. of samples microarray NAGM-GM |
|--------|-----|-----------------------------------------------------|---------------|---------|-----------------------|---------|----------------------------|-----------------------------------|
|        |     |                                                     |               |         |                       |         |                            |                                   |
| Control samples                       |     |                                                     |               |         |                       |         |                            |                                   |
| C01    | M   | Myocardial infarction                               | 8             | 70      |                       | Others  | 1                          |                                   |
| C02    | M   | Cardiac failure and pneumonia                       | 14            | 65      |                       | Others  | 5                          |                                   |
| C04    | F   | Acute pancreatitis                                  | 20            | 58      |                       | 15:01   | 6                          |                                   |
| C07    | M   | Rectal cancer and pneumonia                         | 9             | 89      |                       | Others  | 1                          |                                   |
| C09    | F   | Pneumonia                                           | 10            | 95      |                       | 15:01   | 2                          |                                   |
| C10    | F   | Esophagus cancer                                    | 9             | 85      |                       | Others  | 3                          |                                   |
| C11    | F   | Bronchopneumonia and cerebrovascular accident       | 9             | 93      |                       | Others  | 1                          |                                   |
| C13    | M   | Cardiogenic shock                                   | 21            | 73      |                       | Others  | 4                          | 2                                 |
| C14    | M   | Lung cancer, metastasized                           | 26            | 77      |                       | Others  | 3                          | 3                                 |
| C15    | M   | Myocardial infarction                               | 18            | 64      |                       | Others  | 4                          | 4                                 |
| C17    | F   | Congestive cardiac failure                           | 24            | 84      |                       | Others  | 4                          | 3                                 |
| C18    | M   | Carcinoma of the tongue                             | 22            | 35      |                       | 15:01   | 6                          | 4                                 |
| C20    | F   | Ovarian cancer                                       | 13            | 60      |                       | Others  | 5                          | 5                                 |
| C21    | M   | Cerebrovascular accident and pneumonia              | 17            | 75      |                       | Others  | 4                          | 9                                 |
| C22    | M   | Prostate cancer, metastasized                       | 22            | 88      |                       | Others  | 1                          |                                   |
| C25    | M   | Bladder cancer and pneumonia                        | 5             | 84      |                       | Others  | 3                          | 4                                 |
| C26    | F   | Breast cancer, metastasized                         | 12            | 87      |                       | Others  | 1                          | 2                                 |
| C27    | M   | Renal failure and multiple myeloma                  | 24            | 75      |                       | Others  | 1                          | 2                                 |
| C28    | F   | Cardiac failure                                     | 21            | 60      |                       | 15:01   | 4                          |                                   |
| C29    | M   | Pneumonia                                           | 20            | 60      |                       | Others  | 4                          |                                   |
| C30    | M   | Pericardial tamponade                               | 7             | 68      |                       | Others  | 4                          |                                   |
| C31    | M   | Anaphylaxis                                         | 14            | 72      |                       | 15:01   | 4                          |                                   |
| C32    | F   | Pneumonia                                           | 16            | 71      |                       | 15:01   | 4                          |                                   |
| C33    | F   | Cardiac failure                                     | 12            | 83      |                       | Others  | 4                          |                                   |
| C37    | F   | Pneumonia                                           | 14            | 72      |                       | Others  | 4                          |                                   |
| C38    | F   | Pneumonia                                           | 3             | 88      |                       | Others  | 2                          |                                   |
| C39    | M   | Acute cardiac death                                 | 10            | 69      |                       | 15:01   | 6                          |                                   |
| C44    | F   | Multiorgan failure                                  | 9             | 72      |                       | 15:01   | 4                          |                                   |
| C45    | M   | Cardiopulmonary degeneration and prostate cancer    | 22            | 77      |                       | Others  | 1                          | 1                                 |
| C47    | M   | Cardiac failure and acute erosive enteritis         | 15            | 53      |                       | Others  | 2                          |                                   |
| C48    | M   | Asphyxia                                            | 11            | 61      |                       | 15:01   | 2                          |                                   |

Continued
Table 1 Patient data (continued)

| Cases | Sex | Cause of death                              | p.m. time (h) | Age (a) | Disease duration (a) | MS type | HLA-DRB1* 15:01 | No. of brain tissue blocks | No. of samples microarray NAGM-GM |
|-------|-----|---------------------------------------------|---------------|---------|----------------------|---------|-----------------|----------------------------|---------------------------------|
| C49   | F   | Cardiac failure                            | 16            | 77      |                      | Others  | 2               |                            |                                 |
| C50   | M   | Cardiac failure                            | 21            | 75      | 15:01                | 2       |                 |                            |                                 |

14 F   19 M
Ø = 15.0 Ø = 73.2
10 15:01
23 others
104 42

| Cases | Sex | Cause of death                              | p.m. time (h) | Age (a) | Disease duration (a) | MS type | HLA-DRB1* 15:01 | No. of brain tissue blocks | No. of samples microarray NAGM-GM |
|-------|-----|---------------------------------------------|---------------|---------|----------------------|---------|-----------------|----------------------------|---------------------------------|
| M01   | F   | Breast cancer and pneumothorax             | 8             | 56      | 31                   | SPMS    | Others          |                            |                                 |
| M02   | F   | Peritonitis                                 | 16            | 58      | 22                   | PPMS    | Others          |                            |                                 |
| M03   | F   | NA                                          | 18            | 78      | 14                   | SPMS    | Others          |                            |                                 |
| M04   | F   | Respiratory failure                         | 21            | 42      | 18                   | SPMS    | 15:01           | 1                          |                                 |
| M05   | F   | Sepsis                                      | 19            | 74      | 26                   | SPMS    | 15:01           | 1                          |                                 |
| M06   | F   | Pneumonia                                   | 6             | 58      | 21                   | PPMS    | 15:01           | 6                          | 10                              |
| M07   | F   | Pulmonary embolus and pneumonia             | 17            | 45      | 20                   | PPMS    | Others          |                            |                                 |
| M09   | M   | Aspiration pneumonia                        | 8             | 75      | 38                   | SPMS    | Others          |                            |                                 |
| M10   | F   | Pneumonia                                   | 8             | 72      | 41                   | SPMS    | Others          |                            |                                 |
| M11   | M   | Pneumonia                                   | 26            | 66      | 31                   | SPMS    | Others          |                            |                                 |
| M12   | F   | NA                                          | 11            | 69      | 31                   | NA      | 15:01           | 3                          |                                 |
| M13   | M   | Pneumonia                                   | 11            | 63      | 39                   | SPMS    | Others          |                            |                                 |
| M14   | F   | Pneumonia                                   | 9             | 77      | 31                   | PPMS    | Others          |                            |                                 |
| M15   | F   | MS                                          | 15            | 51      | 21                   | SPMS    | Others          |                            |                                 |
| M16   | F   | Adenocarcinoma of unknown primary           | 6             | 56      | 17                   | PRMS    | 15:01           | 1                          | 1                               |
| M17   | F   | Respiratory infection                       | 10            | 49      | 19                   | SPMS    | Others          |                            |                                 |
| M18   | F   | Pneumonia                                   | 13            | 66      | 30+                  | NA      | 15:01           | 3                          |                                 |
| M20   | F   | Pneumonia                                   | 21            | 86      | 56                   | NA      | Others          |                            |                                 |
| M21   | F   | Pneumonia                                   | 11            | 86      | 36                   | SPMS    | Others          |                            |                                 |
| M22   | F   | MS                                          | 21            | 77      | 22                   | PPMS    | 15:01           | 1                          | 1                               |
| M23   | F   | Lung cancer, metastasized                   | 5             | 78      | 42                   | SPMS    | 15:01           | 4                          | 5                               |
| M24   | F   | Renal failure                               | 31            | 49      | 18                   | SPMS    | Others          |                            |                                 |
| M26   | F   | Bowel blockage and heart failure            | 24            | 71      | 35                   | SPMS    | 15:01           | 1                          |                                 |
| M27   | F   | Pneumonia                                   | 9             | 49      | 25                   | SPMS    | Others          |                            |                                 |
| M28   | F   | Pneumonia                                   | 22            | 54      | 20                   | SPMS    | 15:01           | 3                          |                                 |
| M30   | F   | Pneumonia                                   | 7             | 77      | 21                   | SPMS    | Others          |                            |                                 |
| M31   | M   | Urinary tract infection and MS              | 12            | 53      | 11                   | SPMS    | Others          |                            |                                 |

Continued
BeadChips were scanned by the iScan Array scanner (Illumina). All subsequent data analyses were performed using the statistical software R (R core development Team 2008; R version 3.5.0). Specifically, the Bioconductor packages beadarray (version 2.30.0) and illuminaHumanWGDASLv4.db (version 1.26.0) were used for reading-in data files and for probe annotation (probes n = 48,107). Between-array normalization was performed by variance stabilizing transformation followed by a quantile normalization using functions from the Bioconductor package lumi (version 2.32.0). Only probes mapping to an ENTREZ gene ID were retained. Probes with quality status “bad” were removed. “Bad quality probes” are probe matches repeat sequences, intergenic or intronic regions, or is unlikely to provide specific signal for any transcript (according to illuminaHumanWGDASLv4 annotation). Because the resulting probes (n = 25,081) were still not unique, we selected the probe with the highest variance across all samples, neglecting sample values, which fall into the expression range of negative control probes. This way, each gene is

### Table 1 Patient data (continued)

| Cases | Sex | Cause of death          | p.m. time (h) | Age (a) | Disease duration (a) | MS type       | HLA-DRB1*15:01 | No. of brain tissue blocks | No. of samples microarray NAGM-GM |
|-------|-----|-------------------------|---------------|---------|----------------------|---------------|---------------|-----------------------------|----------------------------------|
| M32   | F   | Pneumonia               | 18            | 39      | 21                   | PRMS          | 15:01         | 1                           | 4                                |
| M33   | M   | Pneumonia               | 19            | 38      | 17                   | PRMS          | 15:01         | 2                           | 1                                |
| M34   | M   | NA                      | 9             | 92      | 54                   | PPMS          | 15:01         | 2                           |                                  |
| M36   | M   | Pneumonia               | 16            | 44      | 16                   | SPMS          | 15:01         | 2                           |                                  |
| M40   | M   | Respiratory failure     | 10            | 40      | 9                    | SPMS          | 15:01         | 3                           |                                  |
| M42   | F   | MS                      | 12            | 50      | 31                   | SPMS          |               | 2                           |                                  |
| M43   | F   | Pneumonia               | 12            | 34      | NA                   | SPMS          | 15:01         | 1                           |                                  |
| M44   | F   | Small bowel obstruction | 13            | 80      | 36                   | SPMS          |               | Others                      | 2                                |
| M46   | F   | Multiorgan failure and sepsis | 28            | 45      | 6                    | SPMS          | 15:01         | 2                           | 3                                |
| M47   | M   | Intestinal obstruction  | 12            | 37      | 27                   | PPMS          |               | 2                           |                                  |
| M48   | F   | Pneumonia               | 24            | 78      | 47                   | SPMS          |               | Others                      | 1                                |
| M51   | F   | Pneumonia               | 12            | 59      | 27                   | PPMS          |               | Others                      | 3                                |
| M52   | M   | Pneumonia               | 24            | 45      | 25                   | SPMS          | 15:01         | 1                           |                                  |
| M53   | F   | Sepsis and pneumonia    | 16            | 47      | 17                   | SPMS          | 15:01         | 4                           | 4                                |
| M54   | M   | MS                      | 9             | 45      | 18                   | SPMS          | 15:01         | 1                           |                                  |
| M55   | F   | MS                      | 26            | 37      | 17                   | SPMS          |               | Others                      | 2                                |
| M56   | F   | Pneumonia               | 22            | 88      | 32                   | PPMS          | 15:01         | 1                           | 2                                |
| M57   | F   | COPD                    | 17            | 58      | 16                   | PPMS          |               | Others                      | 2                                |
| M58   | F   | MS                      | 7             | 80      | 37                   | SPMS          | 15:01         | 1                           |                                  |
| M59   | F   | MS                      | 13            | 42      | 11                   | SPMS          | 15:01         | 2                           |                                  |
| M60   | F   | Respiratory failure     | 9             | 59      | 4                    | PPMS          | 15:01         | 2                           | 3                                |
| M61   | M   | Pancreatic cancer       | 10            | 61      | 26                   | SPMS          | 15:01         | 1                           | 2                                |
| M37   | F   |                          | 14            | 45      | 18                   | SPMS          |               | Others                      | 2                                |

Abbreviations: COPD = chronic obstructive pulmonary disease; GM = control gray matter; NA = not available; NAGM = normal-appearing cortical gray matter; p.m. time = postmortem time (hours); PPMS = primary progressive multiple sclerosis; PRMS = progressive-relapsing multiple sclerosis; SPMS = secondary progressive multiple sclerosis.

The table shows the patient characterization and the use of the patient samples in the different experiments. A more detailed table is given in the supplements (table e-1, links.lww.com/NXI/A173). HLA-DRB1*15:01 is highlighted in bold.
Figure 1 Tissue processing for microarray and tissue characterization

(A) Flowchart to illustrate the process from the patient’s death to statistical analysis of the gene expression microarray. After dissection of the brain and exclusion of confounding pathologies, the tissue blocks were sent to Basel, Switzerland. There, an immunohistochemical characterization was performed, any tissue with bad preservation was excluded, and regions of interest were selected. After RNA isolation, the RIN was measured, and samples with RIN smaller than 6 were excluded. Sample mix up was checked by wrong sex and by principal component analysis.

(B) Representative images of control cortical gray matter (case C30) and MS NAGM (case MS08, asterisk delineates the meninges; I–VI indicate the 6 neuronal layers). NAGM was defined as no loss of MOG, NeuN, or OLIG2 (inset, arrows) staining compared with control cases and no increase in CD68 compared with controls; i.e., occasional CD68+ staining intra- or perivascular (inset, arrowheads) and nearly no CD68+ staining in the tissue. Scale bars: 250 μm; inset Olig2: 20 μm, inset CD68: 10 μm. IHC = immunohistochemistry; MOG = myelin oligodendrocyte glycoprotein; NAGM = normal-appearing cortical gray matter; NeuN = neuronal nuclei; OLIG2 = oligodendrocyte transcription factor 2; PCA = principal component analysis; RIN = RNA integrity index.

represented by the probe, which contains most information on potential expression differences, but ignores probes, which appear artificially regulated due to false-negative regulation introduced by single nucleotide polymorphisms (SNPs). This strategy gave rise to 17,908 unique gene-level probes.
**HLA genotyping**

Human leukocyte antigen (HLA) genotyping was performed by Histogenetics (NY). Allelic variants were typed by sequencing at high resolution (3-field). Alleles bearing suffix “G” in the A locus have identical sequences in exon 2 and exon 3 antigen recognition sites. Alleles bearing suffix “G” in the DRB locus have identical sequences in exon 2 antigen recognition sites. Genotypes are shown in table e-3 (links.lww.com/NXI/A173).

**Immunofluorescence colocalization**

Immunofluorescent colocalization was performed as described before (table e-2A, links.lww.com/NXI/A173).8,10 As tissue preservation is not optimal in fresh-frozen tissues, further paraffin-embedded tissue blocks were stained for colocalization. Paraffin-embedded tissue sections (2–3 μm) were deparaffinized in xylene, rehydrated, and transferred to 3% H2O2 in phosphate buffered saline (PBS) for 20 minutes at 4°C to block the endogenous peroxidase. After 3 washing steps with PBS, the sections were incubated with blocking buffer (10% fetal calf serum in PBS) for at least 20 minutes to reduce unspecific antibody binding. Primary antibodies (table e-2A) were diluted in blocking buffer and incubated overnight at 4°C and then washed 3 times with PBS. Secondary antibodies were incubated for 1–2 hours (table e-2A).

**Histologic quantification**

HLA-DRB1 protein expression was measured with Fiji (image processing package including Imagej)15 using the count objects algorithm with the following parameters: lower threshold 0, upper threshold 120 in green channel of red-green-blue color space, size of objects 10 μm2–100 μm2, and circularity 0.25–1.00. Cortical lesions were defined as areas with complete loss of anti-MOG staining or areas with reduced myelin density clearly demarcated from surrounding normal-appearing tissue. Only cortical gray matter ranging from the white matter to the meninges and with all 6 neuronal layers visible in the adjacent NeuN staining was used. NAGM and gray matter lesion areas were outlined in Adobe Photoshop CS6 (version 13.0 x64; Adobe Systems, CA), exported, and evaluated for area (in squared millimeters) in Imagej software (references 13 and 14, version 1.51s, Fiji distribution, NIH, Maryland). A schematic drawing is shown in figure 4C.

**Statistical analysis**

All statistical analyses were performed using R.15 A p value or false detection rate-adjusted p value smaller than 0.05 was considered statistically significant. Expression data were analyzed using R and the Bioconductor package limma (version 3.36.5). Statistical analysis was performed using a linear model with disease group and sex as factors. Because some patients contributed multiple tissue samples (tissue blocks), we additionally distinguished these “technical” replicates from true biological replicates (patients) in the model to avoid a potential inflation of significance by pseudoreplication. Specifically, the duplicateCorrelation function of the limma package was used to estimate a consensus correlation between technical replicates and this value together with patient ID as a block factor entered into the model fit function. To test the correlation between HLA-DRB1 and HLA-DRA gene expression, a linear model was used (figure 2J). To test the influence of the HLA-DRB1*15:01 genotype and the HLA-DRB1 gene expression on the demyelinated gray matter lesion area per total gray matter area, p values were derived from a linear model weighted by number of tissue blocks per patient (figure 4D). For all other statistical tests, a 2-sided Welch t test was used.

**Data availability**

The gene expression data discussed in this publication have been deposited in NCBI’s Gene Expression Omnibus,16 accession number GSE131282,.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE131282.

**Results**

**HLA-DRB1 is significantly upregulated in MS NAGM compared with control GM**

We investigated the gene expression in MS NAGM and control cases. All tissues were characterized histopathologically, and tissues with signs of possible confounding pathologies were excluded (figure 1A). As a result, only tissues without signs of demyelination, neuronal degeneration, oligodendrocyte loss, and without signs of inflammation such as microglia activation and macrophage infiltration were included in the microarray study (figure 1B). Differential gene expression analysis between MS NAGM and control GM revealed HLA-DRB1 as the most significant differentially regulated gene (figure 2A) (fold-change [FC] = 4.62, adj. p value = 0.013). Besides HLA-DRB1, we detected a trend toward upregulation for the integrin subunit beta-1-binding protein 1 (ITGB1BP1), protein kinase cAMP-dependent type 1 regulatory subunit alpha (PRKAR1A), long intergenic nonprotein coding RNA 115 (LINC00115), mitogen-activated protein kinase kinase 7, also known as TGFi (MAP3K7), and SLIT-ROBO Rho GTPase activating protein 2 (SRGAP2) (figure 2A).

Further analysis of HLA-DRB1 expression showed that the distribution of HLA-DRB1 expression was bimodal within both the MS and the control group (figure 2B). Of special interest is that the majority of individuals with high HLA-DRB1 expression were in the MS group. As HLA genes often show tight linkage disequilibrium patterns,17 we further investigated the expression of DRB5, DQA1, and DQB1, as certain alleles of these genes were reported to form a tight linkage group within the DR15 haplotype.18 The MS-associated DR15 haplotype consists of 5 alleles, namely DRA*01, DRB1*15:01, DRB5*01:01, DQA1*01:02, and DQB1*06:02. Although not significantly differentially expressed between MS and control cases (FC = 4.45, adj. p value = 0.529), we also identified a bimodal distribution for HLA-DRB5 (figure 2C). HLA-DQA1 and HLA-DQB1 expression levels were normally distributed (figure 2, D and E), with HLA-DQB1 expression at the lower detection limit.
Figure 2 Differential gene expression in MS NAGM vs control case cortical gray matter

(A) Volcano plot of the differential gene expression analysis between MS NAGM and control gray matter (GM) revealed HLA-DRB1 as the most significant differentially regulated gene (FC = 4.62, adj. p value = 0.013, marked in red). Differentially expressed genes with an adjusted p value between 0.05 and 0.1 are marked in black. This was the case for ITGB1BP1 (FC = 1.67, adj. p value = 0.065), PRKAR1A (FC = 1.93, adj. p value = 0.065), LINC00115 (FC = 1.5, adj. p value = 0.065), MAP3K7 (FC = 1.34, adj. p value = 0.067), and SRGAP2 (FC = 1.5, adj. p value = 0.067). Boxplots show the log2 gene expression of HLA-DRB1 (B), HLA-DRB5 (C), and HLA-DQA1 (D) and HLA-DQB1 (E) between MS NAGM and GM. HLA-DRB1 is significantly differentially expressed between MS NAGM and GM (B).

Both, HLA-DRB1 (B) and HLA-DRB5 (C) show a bimodal expression pattern in MS and control tissue, whereas the expression of HLA-DQA1 (D) was normally distributed within the sample groups, and HLA-DQB1 (E) was at the lower detection limit. (F) Boxplots show log2 of the HLA-DRB1 gene expression in all tissue samples and in all cases used for the microarray analysis. All cases with the HLA-DRB1*15:01 genotype show a high HLA-DRB1 expression (red dots).

(G) HLA-DRB1 expression between samples from cases carrying the HLA-DRB1*15:01 or other HLA-DRB1 alleles. Boxplot shows that all HLA-DRB1*15:01 tissue samples belong to the HLA-DRB1 high expresser group. (H) HLA-DRB3, 4, and 5 expression of all samples. (I) Comparison of hetero- and homozygote carriers of the HLA-DRB1*15:01 allele. (J) Correlation of HLA-DRB1 with HLA-DRA within the HLA-DRB1*15:01 positive cases. (H and I) p Values are derived from a 2-sided Welch t test. (J) p Value is derived from a linear model.
High cortical HLA-DRB1 expression is associated with HLA-DRB1*15:01 haplotype

The bimodal HLA-DRB1 expression pattern prompted us to investigate whether HLA-DRB1 expression in all tissue samples from 1 individual shows this mode of expression. This analysis revealed that single cases either expressed HLA-DRB1 at high or low levels in both MS and control cases (figure 2F). As HLA-DRB1*15:01 is strongest associated with MS risk\(^1\),\(^2\) and HLA-DRB5 showed a similar expression distribution, we genotyped all cases for HLA-DRB1, 3, 4, and 5 at a 3-field resolution (table e-3, links.lww.com/NXI/A173). As expected, we detected a trend toward higher frequency of the HLA-DRB1*15:01 allele among the MS cases compared with control cases (Fisher exact test, \(p = 0.083\), OR = 4.5, 95% CI = [0.8–50.2]).\(^3\)\(^4\) Gene expression analysis based on the HLA-DRB1 genotype revealed that the bimodal distribution was linked to the HLA-DRB1 genotype with individuals with the HLA-DRB1*15:01 allele always showing high transcriptional expression of HLA-DRB1 (\(n = 106\) samples, figure 2, F and G). In contrast to HLA-DRB1, which is expressed in every case, only 11 MS and 1 control case turned out to carry the HLA-DRB5 gene (table e-3). As expected, all individuals genotyped positively for HLA-DRB5*01:01 allele were also positive for HLA-DRB1*15:01 (table e-3). Compared with HLA-DRB3 and -DRB4 alleles in other DR haplotypes, HLA-DRB5*01:01 always showed a higher expression (\(p < 0.001\), \(df = 79.33\), for DRB3, figure 2H; \(p < 0.001\), \(df = 89.58\) for DRB4, figure 2H). Notably, there were no significant differences in HLA-DRB1 gene expression levels between hetero- and homozygotic carriers of the HLA-DRB1*15:01 allele (\(p = 0.379\), \(df = 22.01\), figure 2I). Beside the cases carrying the HLA-DRB1*15:01 allele, 5 MS and 3 control cases also showed high HLA-DRB1 expression (figure 2F). Of these cases, one MS case was heterozygote for the HLA-DBR1*04:01 allele, and another case was heterozygote for the HLA-DRB1*08:01:01G allele. Of interest, both alleles have been associated with risk of MS.\(^1\),\(^2\)\(^\text{19}\),\(^\text{20}\) All 3 control cases were heterozygote for the HLA-DRB1*03:01 allele, also previously shown to be associated with MS\(^19\),\(^\text{20}\) (table e-3, risk genes marked in bold). Of the other 3 MS cases with high HLA-DRB1 expression, 2 did not carry a MS-associated allele (HLA-DRB1*01:01, *01:01:01G, *01:03, and *07:01:01G), and in 1 case, genotyping failed.

We did not detect any systematic differences between the HLA-DRB1*15:01 cases compared with the other cases concerning age at death, age at disease onset, and disease duration (figure e-1, A–C, links.lww.com/NXI/A173). Also, we did not detect any differences between the HLA-DRB1 high expressing cases compared with the low expressing cases concerning age at death, age at disease onset, and disease duration (figure e-1, D–F).

High HLA-DRB1 expression correlates with high expression of HLA-DRA

Functional HLA-DR molecules are heterodimers of a DRA-encoded alpha chain and a beta chain encoded by DRB1 or DRB3,4,5, respectively. Therefore, we investigated whether high HLA-DRB1 expression correlates with high HLA-DRA gene expression in HLA-DRB1*15:01 carriers. Indeed, high HLA-DRB1 gene expression correlated with high HLA-DRA (\(r = 0.79\), \(p < 0.001\)) gene expression in HLA-DRB1*15:01 cases, supporting the idea of a biologically functional upregulation of MHCII in MS NAGM of HLA-DRB1*15:01 cases (figure 2J).

HLA-DRB1 is expressed by microglia in human cortical gray matter

To determine which cell types are expressing HLA-DRB1 in NAGM, a confocal immunofluorescence colocalization analysis of fresh-frozen and paraffin-embedded human brain tissues was performed (figure 3). We detected that HLA-DRB1 colocalized with microglia in MS NAGM and control GM (figure 3A). No colocalization could be detected in astrocytes (figure 3B), neurons (figure 3C), oligodendrocytes (figure 3D), or blood vessels (figure 3E).

HLA-DRB1 protein expression is elevated in HLA-DRB1*15:01-positive cases

Quantitative immunohistochemical analysis was performed to determine whether HLA-DRB1 gene and protein expression are associated with each other (figure 4A). We detected a higher protein expression in high vs low HLA-DRB1 gene expressers (\(p = 0.052\), \(df = 46.9, n = 49\)) (figure 4B) and a trend toward higher expression in MS and control cases carrying the HLA-DRB1*15:01 allele compared with non-carriers (\(p = 0.097, df = 60.5, n = 74\)). HLA-DRB1 protein expression varied considerably from case to case (figure 4B).

HLA-DRB1*15:01 genotype and increased expression of HLA-DRB1 is associated with increased cortical demyelination

We hypothesized that the HLA-DRB1*15:01 genotype and the increased HLA-DRB1 gene- and protein expression might be related to meningeal inflammation and cortical microglia activation, previously described in MS,\(^2\)\(^3\) and hence might also correlate with increased levels of cortical demyelination.

We thus investigated all available tissue blocks from MS cases with cortical lesions (table e-1, links.lww.com/NXI/A173) and stained for MOG, delineated cerebral cortical area containing all 6 layers (identified by NeuN) (figure 4C), and quantified the fraction of demyelinated vs whole cortical area (figure 4D). Areas of demyelination in MS cases carrying the HLA-DRB1*15:01 allele, and respectively cases with a high HLA-DRB1 gene expression, were significantly larger than in HLA-DRB1*15:01-negative cases (\(p = 0.052, df = 23, n = 25\)) and, respectively, cases with low HLA-DRB1 gene expression (\(p = 0.014, df = 21, n = 23\)) (figure 4D).

HLA-DRB1*15:01 carrier status associates with higher expression of 9 genes in cortical NAGM in both MS and controls

We investigated the effect of the HLA-DRB1*15:01 allele by analyzing the differential gene expression data after grouping...
the cases into DRB1*15:01 positive or negative. We detected 9 genes to be differentially regulated. Most interestingly, our data show an upregulation of interleukin 18 receptor 1 (IL18R1; FC = 1.73, adj. p value = 0.004) and leukocyte immunoglobulin like receptor B1 (LILRB1; FC = 1.54, adj. p value = 0.032). The highest fold change was detected for long intergenic nonprotein coding RNA 01119 (LINC01119, FC = 4.35, adj. p value < 0.0001). We further detected differentially expressed transcripts of protein O-fucosyltransferase 2 (POFUT2; FC = 1.86, adj. p value < 0.001), G protein subunit beta 5 (GNB5; FC = -2.20, adj. p value = 0.003), epithelial stromal interaction 1 (EPSTI1; FC = 1.50, adj. p value = 0.005), DExD/H-box helicase 60 (DDX60; FC = 1.25, adj. p value = 0.010), N-acetylneuraminic acid phosphatase (NANP; FC = 1.47, adj. p value = 0.012), and kinesin family member 25 (KIF25; FC = -1.37, adj. p value = 0.049).

**Discussion**

Our results demonstrate that HLA-DRB1 is significantly higher expressed in MS NAGM and shows a bimodal distribution with more MS cases showing a high expression compared with control cases. Genotyping of the HLA locus revealed an almost exclusive high expression of all HLA-DRB1*15:01 allele carriers and a few other MS-associated risk alleles. Consistent with the gene expression analysis, HLA-DRB1 protein expression is increased in HLA-DRB1*15:01-positive cases in gray matter on microglia based on immunofluorescence colocalization. The HLA-DRB1*15:01 genotype and high HLA-DRB1 gene expression are associated with larger gray matter lesions in MS. Furthermore, it is important to note that the second DR allele, DRB5*01:01, which is tightly linked with DRB1*15:01 in the MS-associated DR haplotype is also expressed at higher levels.

These findings hint at a link between the strongest genetic risk factor for MS and an important pathologic hallmark, namely demyelinated lesions in the cerebral cortex. We hypothesize that the HLA-DRB1*15:01 genotype and the increased HLA-DRB1 and-DRB5 gene expression together with meningeal and parenchymal inflammation may lead to larger demyelinated lesions. The correlation between inflammation, cortical demyelination, and the HLA-DRB1*15 allele has already been described in autopsy tissue.
by Yates et al., who have detected more parenchymal, perivascular, and meningeal T-cell inflammation and larger motor cortical lesions in HLA-DRB1*15 carriers compared with carriers of other alleles. Although MRI detection of cortical gray matter lesion is improving, widespread subpial demyelination is still difficult to detect. A recent MRI study of 85 patients with MS did not reveal a statistically significant difference between HLA-DRB1*15:01 carriers and non-carriers; however, the authors point out the limited power due to the small number of cases. Furthermore, meningeal inflammation and follicle-like structures in the meninges have been linked to microglia activity and larger subpial cortical gray matter lesions. Regarding expression of the 2 DR15 alleles, higher messenger RNA expression of
DRB5*01:01 compared with DRB1*15:01 in MS lesions and normal-appearing white matter have already been observed previously supporting our findings; however, much fewer cases and neither gray matter nor the extent of demyelination had been studied.  

HLA class II molecules present processed peptides to CD4+ T lymphocytes. In MS, the strongest genetic association maps to the HLA-DRB1 gene, whereas the association with DRB5*01:01 has so far largely been ignored because the SNPs, which have been used for determining DR types from SNP typing, are not sufficiently tightly spaced in the HLA region on chromosome 6 to allow assignment of the specific HLA-DR3*, -4*, and -5* alleles. The HLA-DRB1*15:01 allele was reported to increase the risk for developing MS about threefold. The higher HLA-DRB1 expression in the cortical gray matter in HLA-DRB1*15:01 cases may therefore be involved in local activation of autoreactive CD4+ T cells. For CD4+ T-cell activation to occur, an interaction between the T-cell receptor and major histocompatibility complex (MHC) class II/peptide (pMHC) complexes is required. The amount of antigen loaded on MHC class II molecules of antigen-presenting cells and the level of MHC class II expression determine the activation of CD4+ T-cell activation, and higher pMHC ligand densities enhance this process. Similar to the ectopic expression of HLA class II in autoimmune thyroid disease, higher HLA-DRB1 expression by microglia in the brain may therefore affect the pMHC concentration and consequently lead to an increased probability of T-cell activation and brain inflammation. This hypothesis is further supported by the finding that the level of HLA-DR expression in transgenic mice is an important prerequisite for developing spontaneous experimental autoimmune encephalomyelitis.

Besides their expression levels, the nature of the peptide repertoire that is presented by the 2 HLA-DR alleles in the MS-associated DR15 haplotype, i.e., DRB1*15:01 and DRB5*01:01, probably also plays an important role for the activation of autoreactive T cells. Finally, autoreactive- and virus-specific, brain-infiltrating CD4+ T cells may recognize peptides in the context of both DR alleles of the DR15 haplotype, indicating that the expression of the 2 DR molecules may increase the likelihood of T-cell activation further.

A bimodal expression pattern of HLA-DRB1 and -DRB5 has been described in lymphoblastoid cell lines. The authors analyzed expression quantitative trait loci in the tag of the HLA-DRB1*15:01 allele associating with high HLA-DRB1, DRB5, and DQB1 gene expression. They concluded that a higher gene expression alone does not sufficiently explain the MS-associated risk of the HLA-DRB1*15:01 allele. At present, it is not clear which molecular pathomechanisms are responsible for the high vs lower expression in some individuals, but differences in gene regulation, for instance by different activation of the class II transactivator, is one possibility.

Regarding the potential functional involvement of the DRB1*15:01 haplotype, the higher DRB1- and DRB5 expression in MS brains raises questions beyond peptide presentation to autoreactive T cells. The brain is considered an immune privileged site, and besides shielding of the CNS from the peripheral immune system via specialized barriers, low MHC expression is considered an important aspect of this CNS immune privilege. Aberrant and increased expression of HLA-DRB1 and -DRB5 may contribute to breaking immune tolerance in this tissue that is exquisitely vulnerable to damage and endowed with cells that are terminally differentiated and not replaceable. The interpretation of recent genome-wide association studies in MS, which found almost exclusively immune system-related genes, has been that MS develops from outside, i.e., the peripheral immune system, in as opposed to starting by damage within the CNS and then involving peripheral immune cells, i.e., inside-out hypothesis. Our data, although preliminary and not addressing functional aspects in human brain tissue, might indicate that increased HLA-DR expression in the brain, if it preceded peripheral immune T-cell activation, could play a role both within and later also outside the brain.

The gene expression analysis further revealed 9 genes to be differentially expressed in HLA-DRB1*15:01-positive vs -negative samples. Among the detected genes, IL18R1 gene and protein expression have previously been shown to be elevated in MS in CSF and peripheral blood mononuclear cells compared with controls. The highest fold change was detected for LINCO1119, a long intergenic non–protein-coding RNA. Research on noncoding RNAs is rapidly evolving, and some members have already been shown to play a role in immune system regulation. We further detected LILRB1, a receptor for class I MHC antigens expressed by different leukocyte lineages that may downregulate monocyte activation signals. EPSTI1 and DDX60 have previously been shown to be differentially regulated on interferon signaling in HLA-B27-transgenic rats, an animal model developing spontaneous autoimmune-mediated multisystem inflammatory disease. The other genes we detected were POFTUT2, GN5B, NAP1, and KIF25. To the best of our knowledge, these genes have not been described to date in the context of MS or HLA-DRB as possibly linked to the HLA-DRB1*15:01 allele. The power of this particular analysis is however limited by the sample heterogeneity and needs
further experimental investigation to evaluate the possible impact of the detected differential expression.

In conclusion, our results demonstrate elevated DR expression in the cortical gray matter of a subset of patients with MS positive for the HLA-DR15 haplotype and rarely also other DR types. HLA-DRB1 expression by microglia in the brain might play a role as vulnerability factor to develop or sustain MS. Further studies on DR expression in the brain, its causes, and consequences would therefore be of great interest for a better understanding of MS pathogenesis.

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Increased HLA-DR expression and cortical demyelination in MS links with HLA-DR15
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