doi:10.1093/brain/awu405 BRAIN 2015: 138; 632–643 | 632

BRAIN
A JOURNAL OF NEUROLOGY

Genetic variants are major determinants of CSF antibody levels in multiple sclerosis

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Immunological hallmarks of multiple sclerosis include the production of antibodies in the central nervous system, expressed as presence of oligoclonal bands and/or an increased immunoglobulin G index—the level of immunoglobulin G in the cerebrospinal fluid compared to serum. However, the underlying differences between oligoclonal band-positive and -negative patients with multiple sclerosis and reasons for variability in immunoglobulin G index are not known. To identify genetic factors influencing the variation in the antibody levels in the cerebrospinal fluid in multiple sclerosis, we have performed a genome-wide association screen in patients collected from nine countries for two traits, presence or absence of oligoclonal bands ($n = 3026$) and immunoglobulin G index levels ($n = 938$), followed by a replication in 3891 additional patients. We replicate previously suggested association signals for oligoclonal band status in the major histocompatibility complex region for the rs9271640*A-rs6457617*G haplotype, correlated with HLA-DRB1*1501, and rs34083746*G, correlated with HLA-DQA1*0301 (P comparing two haplotypes $= 8.88 \times 10^{-16}$). Furthermore, we identify a novel association signal of rs9807334, near the *ELAC1/SMAD4* genes, for oligoclonal band status ($P = 8.45 \times 10^{-7}$). The previously reported association of the immunoglobulin heavy chain locus with immunoglobulin G index reaches strong evidence for association in this data set ($P = 3.79 \times 10^{-37}$). We identify two novel associations in the major histocompatibility complex region with immunoglobulin G index: the rs9271640*A-rs6457617*G haplotype ($P = 1.59 \times 10^{-22}$), shared with oligoclonal band status, and an additional independent effect of rs6457617*G ($P = 3.68 \times 10^{-6}$). Variants identified in this study account for up to 2-fold differences in the odds of being oligoclonal band positive and 7.75% of the variation in immunoglobulin G index. Both traits are associated with clinical features of disease such as female gender, age at onset and severity. This is the largest study population so far investigated for the genetic influence on antibody levels in the cerebrospinal fluid in multiple sclerosis, including 6950 patients. We confirm that genetic factors underlie these antibody levels and identify both the major histocompatibility complex and immunoglobulin heavy chain region as major determinants.

Received July 4, 2014. Revised October 2, 2014. Accepted November 16, 2014. Advance Access publication January 23, 2015

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Keywords: multiple sclerosis; CSF; oligoclonal bands; immunoglobulin; genetics

Abbreviations: HLA = human leukocyte antigen; IgG = immunoglobulin G; IGHC = immunoglobulin heavy chain; MHC = major histocompatibility complex; OCB = oligoclonal band; SNP = single nucleotide polymorphism

Introduction

Multiple sclerosis is a neurological disease characterized by inflammation, demyelination and axonal degeneration, and is an important cause of disability in young adults (Compston and Coles, 2008). The aetiology is not known, but both genetic and environmental factors influence disease susceptibility (Compston and Coles, 2008). An association between multiple sclerosis and the human leukocyte antigen (HLA) genes in the major histocompatibility complex (MHC) region was identified early on (Jersild *et al.*, 1972), and was later refined to four independent association signals of which HLA-DRB1*15:01 is the strongest (The International Multiple Sclerosis Genetics Consortium and The Wellcome Trust Case Control Consortium 2, 2011). Recently, knowledge of non-HLA associations in multiple sclerosis has increased extensively and immunologically relevant genes are overrepresented among those mapping close to the identified risk variants (The International Multiple Sclerosis Genetics Consortium and The Wellcome Trust Case Control Consortium 2, 2011; The International Multiple Sclerosis Genetics Consortium, 2013).

An immunological hallmark in multiple sclerosis is the finding of oligoclonal bands (OCBs) and/or increased immunoglobulin G (IgG) index in the CSF (Stangel *et al.*,

2013). OCBs are reported to be observed in 90–95% of patients in Northern Europe, and are composed predominantly of IgG. The IgG index contrasts the CSF/serum IgG ratio to the CSF/serum albumin ratio (Link and Huang, 2006). There are strong indications that antibody levels in the CSF are influenced by genetic factors, as CSF abnormalities are seen in 19% of unaffected siblings of patients with multiple sclerosis as opposed to 4% of healthy unrelated individuals (Haghighi *et al.*, 2000). Also, the OCB positive rates, as well as the IgG index are reported to correlate with ethnicity (Fukazawa *et al.*, 1998; Kikuchi *et al.*, 2003; Rinker *et al.*, 2007; Lechner-Scott *et al.*, 2011; Yoshimura *et al.*, 2014), although not observed in all studies (Berg-Hansen *et al.*, 2013).

The role of the HLA loci in determining OCB status has been highlighted previously (Fukazawa *et al.*, 1998; Kikuchi *et al.*, 2003; Imrell *et al.*, 2006; Idiman *et al.*, 2009; Wu *et al.*, 2009; Romero-Pinel *et al.*, 2011; Leone *et al.*, 2013; Mero *et al.*, 2013; Yoshimura *et al.*, 2014). Most recently, large study populations from Scandinavia and Italy have shown HLA-DRB1*15:01 to be associated with OCB-positive and HLA-DRB1*04:04 with OCB-negative multiple sclerosis (Leone *et al.*, 2013; Mero *et al.*, 2013). Furthermore, the immunoglobulin heavy chain (IGHC) region was recently reported to be highly correlated with IgG index in German and Belgian patients with multiple sclerosis (Buck *et al.*, 2012).

In the present study we aim to further determine the genetic impact on IgG levels in the CSF in multiple sclerosis. We combine genome-wide single nucleotide polymorphism (SNP) data (The International Multiple Sclerosis Genetics Consortium and The Wellcome Trust Case Control Consortium 2, 2011) with available CSF data in a meta-analysis of 3059 patients with multiple sclerosis and replicate top-hits from the screening phase in 3891 additional samples. The combined data set of 6950 patients from nine countries is the largest study population so far to investigate both clinical and genetic factors associated with OCB status and IgG index and we replicate three previously described associations and identify three new genetic differences underlying CSF phenotypes.

Materials and methods

Study populations

The 3059 multiple sclerosis samples included in the screening phase of this study are a subset of the samples that were previously used in a genome-wide association study on multiple sclerosis susceptibility (The International Multiple Sclerosis Genetics Consortium and The Wellcome Trust Case Control Consortium 2, 2011). Eight countries provided OCB status (positive or negative) for 3026 patients in the screening phase, while five countries provided IgG index for 938 patients (Table 1). In the replication phase, 3891 additional multiple sclerosis samples (3842 with OCB status and 2188 with IgG index) were provided by eight countries. The combined screening and replication cohort included OCB status on 6868 patients and IgG index on 3126 patients from the following countries: Australia, Belgium, Denmark, Germany, Italy, Norway, Spain, Sweden and USA (Table 1). For 3044 patients, both OCB status and IgG index were available. For the genetic analyses, 3478 patients with OCB status and 2072 patients with IgG index survived genetic quality control in the replication phase, leading to a final sample size of 6504 for OCB status and 3010 for IgG index in the combined genetic analyses.

CSF analyses

All patients included in this study fulfilled Poser or McDonald multiple sclerosis criteria (Poser *et al.*, 1983; McDonald *et al.*, 2001) or were diagnosed as clinically isolated syndrome [$n = 7$ (0.2%) in screening phase (Germany); $n = 138$ (3.5%) in replication phase (Germany)], based on the combination of (i) a clinical symptom being typical and suggestive for multiple sclerosis; (ii) MRI of the brain and in most cases also of the spinal cord demonstrating typical multiple sclerosis lesions fulfilling the criteria of dissemination in space (Barkhof criteria or Swanton criteria); and (iii) CSF parameters. In most countries recruiting samples to this study, lumbar puncture is done routinely as part of the diagnostic process and is seldom repeated unless the diagnosis is unclear. OCB status is considered positive when more than one OCB was seen in the CSF that was not present in the serum. IgG index is considered positive for values >0.7. In this study the IgG index value was used in quantitative trait statistical analyses after transforming the IgG index ratio by taking the logarithm (base 2) ($\log_2$ IgG).

Clinical analyses

The clinical parameters gender, age at onset, Multiple Sclerosis Severity Score (Roxburgh *et al.*, 2005), disease course and disease duration at lumbar puncture were included in the clinical analyses. Statistical analyses of CSF phenotypes related to demographic and clinical parameters were done in R 2.14.1 (www.r-project.org) using linear regression for IgG index and logistic regression for the binary OCB status. We included covariates in the analyses as indicated.

Screening phase

Quality control and analysis of genetic data provided in the multiple sclerosis genome-wide association study (The International Multiple Sclerosis Genetics Consortium and The Wellcome Trust Case Control Consortium 2, 2011) were performed with Plink v1.07 (Purcell *et al.*, 2007). For OCB status, we used a qualitative trait analysis in a logistic regression with binary outcome (OCB pos/neg). For IgG index, we applied a quantitative trait analysis in a linear regression with allele dosage as independent variable and IgG index as dependent outcome. In the screening phase, gender and the seven main principal components previously determined in this data set (The International Multiple Sclerosis Genetics Consortium and The Wellcome Trust Case Control Consortium 2, 2011) were associated with IgG index and hence added as covariates for IgG index analyses. Evidence of association was tested in samples from each country individually and combined in a fixed-effects meta-analysis (assuming a qualitative trait for OCB and quantitative trait for IgG) over all countries.

Table 1 Demographic characterization of the multiple sclerosis study populations

Country	Female:male ratio	PP (%)	Age at onset, years, mean (range)	MSSS mean (range)	OCB (*n*)	OCB+ (%)	IgG (*n*)	IgG index mean (range)	IgG+ (%)	Disease duration at lumbar puncture, years, median (range)
SCREEN (*n* = 3059)	**2.28**	**13**	**33 (9–60)**	**4.43 (0.03–9.98)**	**3026**	**88**	**938**	**1.05 (0.29–7.27)**	**64**	**1 (−6–49)**
Belgium (*n* = 358)	1.93	13	34 (9–60)	5.31 (0.03–9.98)	356	89	165	1.12 (0.43–5.36)	68	2 (−2–46)
Denmark (*n* = 197)	2.28	2	32 (12–59)	4.30 (0.11–9.76)	189	90	63	1.24 (0.42–7.27)	87	0 (0–39)
Italy (*n* = 565)	1.80	30	33 (10–60)	4.47 (0.04–9.96)	564	90	133	0.90 (0.34–3.19)	46	1 (0–41)
Norway (*n* = 827)	2.56	12	34 (10–59)	5.09 (0.26–9.86)	827	89	NA	NA	NA	3 (0–41)
Spain (*n* = 62)	1.42	41	33 (10–60)	5.57 (0.21–9.83)	62	79	NA	NA	NA	4 (0–20)
Sweden (*n* = 443)	2.52	4	33 (12–57)	3.01 (0.04–9.96)	439	88	355	1.00 (0.3–5)	61	1 (–6–49)
Germany (*n* = 444)	2.67	6	35 (15–58)	4.70 (0.11–9.84)	426	90	222	1.06 (0.29–3.94)	73	4 (0–19)
USA (*n* = 163)	2.70	10	34 (13–60)	5.54 (0.74–9.88)	163	75	NA	NA	NA	3 (0–36)
REP (*n* = 3891)	**2.27**	**10**	**34 (2–72)**	**3.95 (0.03–9.96)**	**3842**	**87**	**2188**	**1.03 (0.06–6.20)**	**64**	**1 (−6–48)**
Australia (*n* = 343)	3.05	10	35 (2–64)	4.08 (0.05–9.88)	343	71	127	0.98 (0.06–3.1)	61	1 (0–48)
Belgium (*n* = 359)	1.92	12	35 (11–72)	4.16 (0.03–9.94)	356	91	243	1.09 (0.37–4.45)	69	1 (0–44)
Denmark (*n* = 274)	1.98	6	32 (14–61)	4.15 (0.04–9.63)	274	93	274	1.27 (0.35–6.20)	77	2 (−1–32)
Italy (*n* = 759)	2.15	8	31 (7–66)	3.25 (0.09–9.86)	759	88	512	0.89 (0.24–2.97)	55	1 (0–35)
Norway (*n* = 1127)	2.49	13	34 (12–68)	4.47 (0.13–9.95)	1127	89	85	1.26 (0.4–5.5)	82	3 (0–38)
Spain (*n* = 242)	1.81	18	32 (13–65)	4.39 (0.15–9.96)	242	81	161	1.10 (0.4–5.53)	65	1 (0–40)
Sweden (*n* = 380)	2.65	12	38 (12–68)	4.97 (0.10–9.87)	380	90	379	1.03 (0.3–3.8)	68	0 (−6–44)
Germany (*n* = 407)	1.99	3	33 (12–64)	3.40 (0.10–9.77)	361	91	407	0.94 (0.37–5.2)	55	0 (−1–25)

PP = primary progressive multiple sclerosis; MSSS = Multiple Sclerosis Severity Score; REP = replication; OCB + = oligoclonal band positive status, IgG + = IgG index > 0.7.

P-values $< 5 \times 10^{-8}$ and $< 10^{-4.5}$ in the screening phase were considered as genome-wide significant and suggestive evidence, respectively, as applied previously (The International Multiple Sclerosis Genetics Consortium and The Wellcome Trust Case Control Consortium 2, 2011). For each lead SNP a conditional analysis of the surrounding 3 Mb region was performed, and SNPs reaching $P < 10^{-4}$ were considered suggestive in this analysis.

Power for OCB analysis was determined mainly by the typical frequency of OCB-negative status in a multiple sclerosis study population. In the screening phase, we had 80% power to detect suggestive evidence ($P < 10^{-4.5}$) for variants with a minor allele frequency of 0.20 and an odds ratio (OR) of 1.6. Power was 80% for variants explaining 2.5% of the variation in the distribution of IgG index seen in patients with multiple sclerosis.

Replication phase

Forty-two SNPs were selected for replication. Of these, 38 SNPs were brought forward to replication based on the results from the screening phase; 32 lead SNPs reaching $P < 10^{-4.5}$, an additional proxy marker for rs6457617 (rs9275224 with $r^2 = 1$), and five SNPs with conditional association signals of $P < 10^{-4}$. Additionally, we added two SNPs that were previously suggested to be associated with OCB status (Leone *et al.*, 2013; Mero *et al.*, 2013) and two SNPs reported to be associated to autoantibody presence in other tissues (Ferreira *et al.*, 2010; Plagnol *et al.*, 2011) and showing association or a trend for association with IgG index in our study. The SNPs were genotyped using Sequenom MassARRAY® iPLEX® technology (Sequenom Inc.). Sequenom MassARRAY® Designer software v3.1 was used to design primers and extension probes. As part of validating the design, a test set of genotyping data from 86 individuals generated with Illumina platforms was included. Seven SNPs were replaced by proxies with moderate to high linkage disequilibrium ($r^2 > 0.63$). For two SNPs, no assay could be designed and for two others genotyping assays failed, therefore a total of 38 SNPs were successfully included in the replication analysis (i.e. 28 lead SNPs and one synonym, five conditional SNPs and four SNPs selected from previous studies) (Supplementary material). Seventy-five nanograms of genomic DNA was used in 5 µl reactions in 384-well plates. The amplified resin-treated DNA was spotted with a Sequenom MassARRAY® Nanodispenser (Sequenom Inc.) on a SpectroCHIP Array. The SpectroCHIP Arrays were analysed using a Sequenom MALDI-TOF mass spectrometer (Sequenom Inc.). Genotyping calls were automatically generated using the MassARRAY® TyperAnalyzer software v4.0 (Sequenom Inc.) and were validated by manual review of the raw mass spectra scatter plots.

Genotyping quality control was performed in samples grouped per country. Samples with > 4/38 missing genotypes (< 89.5% sample success rate) and SNPs with < 95% genotyping success rate were excluded from further analysis. No SNPs deviated from Hardy-Weinberg equilibrium ($P < 10^{-4}$), except for a known multiple sclerosis susceptibility SNP in the MHC region in the Norwegian population. Analysis was performed per country with a linear model including gender as covariate for IgG and a logistic model for OCB, followed by a fixed-effects meta-analysis over all countries. An effect was considered replicated when reaching $P < 0.05$ in the replication phase.

Combined analyses

Analysis was performed by a fixed-effects meta-analysis over all cohorts (screening and replication cohorts per country as described previously). The percentage of the variance in IgG

index explained by variants was calculated by subtracting adjusted r^2 from a full model with that from the baseline linear model in R. Evidence for interaction between variants, defined as deviation from a multiplicative model, was investigated in a linear (IgG index) or logistic (OCB status) regression in R.

Major histocompatibility complex analyses

In the screening phase HLA-A, -B, -C, -DRB1, -DQA1 and -DQB1 genotypes were imputed from SNP data as described previously (Dilthey *et al.*, 2011; The International Multiple Sclerosis Genetics Consortium and The Wellcome Trust Case Control Consortium 2, 2011). The most likely imputed alleles were used as input for testing of association of common HLA alleles with CSF phenotypes using the same regression approach as described for SNPs above. Independent effects of SNPs/haplotypes in the MHC region were investigated with a likelihood ratio test for OCB status or F-test for IgG index comparing a null model and alternative model for phased haplotypes with a minor haplotype frequency of 0.05 including country, seven principal components (screening phase only) and gender as covariates (–chap option in Plink v1.07). The most likely model was obtained by starting with a null model with equal effects for all haplotypes consisting of known and novel replicated MHC signals and allowing separate effects for individual haplotypes, in order of significance, as long as this improved the model with nominal significance.

Multiple sclerosis genetic burden

In the screening phase, a genetic risk score, multiple sclerosis genetic burden, was calculated on the basis of the number of risk alleles weighted by their effect on multiple sclerosis risk according to the method described previously (Gourraud *et al.*, 2011; Harbo *et al.*, 2014). Two scores were calculated, both including the 57 risk SNPs and either including or excluding alleles imputed as above for the four HLA effects (HLA-DRB1*15:01, DRB1*03:01, DRB1*13:03 and HLA–A*02:01) established in our previous genome-wide association study on susceptibility including this data set (The International Multiple Sclerosis Genetics Consortium and The Wellcome Trust Case Control Consortium 2, 2011). A logistic regression analysis with country as covariate was performed for OCB status, whereas a linear regression analysis including country, gender and seven principal components was performed for IgG index in R.

Results

Clinical and CSF analyses

The main demographic and clinical characteristics of patients included in the screening and replication phase, stratified by country of origin, are described in Table 1. Among 6868 patients with multiple sclerosis with known OCB status, 6033 (88%) were OCB-positive. The IgG index level in patients recruited from different countries is shown in Supplementary Fig. 1. An elevated IgG index was found in 1996 of 3126 patients with known IgG index (64%). IgG index was on average lower in Italian and higher in Danish and Norwegian patients. For 3044 patients, both OCB status and IgG index were available. Increased IgG index was highly correlated with OCB status ($P < 2 \times 10^{-16}$) in the combined data set (Table 2 and Supplementary Fig. 2). Overall, 62% of the patients with multiple sclerosis were positive and 10% were negative for both OCB and IgG index (Supplementary Table 1). On average, 26% of the patients were OCB-positive but did not have an increased IgG index. An increased IgG index in OCB-negative patients with multiple sclerosis was rare (2%).

Gender was highly correlated with both IgG index and OCB status. Females had on average a 1.12-fold higher IgG index ($P = 1.9 \times 10^{-10}$) and were 1.3-fold more likely to be OCB-positive than males ($P = 9.7 \times 10^{-4}$) (Table 2 and Supplementary Fig. 3). There was no correlation either between IgG index or OCB status and the interval between onset and lumbar puncture (median of 1 year). Younger patients had higher IgG indices ($P = 0.013$), and were more likely to be OCB-positive ($P = 0.0028$). We found no correlation between multiple sclerosis subtype (bout onset or progressive onset) and IgG index or OCB status after correction for gender and age at onset. Patients with a higher IgG index were more severely affected, as assessed by the Multiple Sclerosis Severity Score ($P = 4.0 \times 10^{-4}$).

Genome-wide association screen

After quality control of the screening data, 485 522 SNPs in 3026 samples and 485 236 SNPs in 938 samples were available for the analysis of OCB status and IgG index, respectively (Supplementary Table 2). Genomic inflation factor was 0.987 for OCB and 1.015 for IgG index (Supplementary Fig. 4).

We first performed a genome-wide analysis of all SNPs and CSF data available (Fig. 1). Markers in the MHC region on chromosome 6 were associated with OCB status with genome-wide significance ($P < 5 \times 10^{-8}$). We identified two regions showing genome-wide significance for association based on the IgG index, the MHC region on chromosome 6 and the IGHC region on chromosome 14. In addition, 14 lead SNPs and two conditional SNPs, and 15 lead SNPs and three conditional SNPs reached suggestive evidence for OCB status and IgG index, respectively (Tables 3 and 4), and were thus taken forward to replication.

When analysing the 57 multiple sclerosis risk SNPs previously identified in the genome-wide association study (The International Multiple Sclerosis Genetics Consortium and The Wellcome Trust Case Control Consortium 2, 2011) (Supplementary Table 3), one SNP (rs12368653) showed nominally significant association with OCB positivity. For IgG index, 7 of these 57 SNPs (12%) reached nominal significance. The multiple sclerosis associated risk allele increased the IgG index for five of these SNPs. As the

Table 2 Correlation of CSF measures with demographic and clinical variables of the included multiple sclerosis patients

	Screening			Replication			Combined		
Log IgG versus	***n***	**Fold change[e]**	***P***	***n***	**Fold change[e]**	***P***	***n***	**Fold change IgG index (95% CI)[e]**	***P***
Gender (female)[a]	938	1.136	**1.4×10^{-4}**	2188	1.115	**7.6×10^{-7}**	3126	1.124 (1.085–1.165)	**1.9×10^{-10}**
OCB-status (positive)[b]	905	1.637	**$<2 \times 10^{-16}$**	2139	1.640	**$<2 \times 10^{-16}$**	3044	1.639 (1.559–1.724)	**$<2 \times 10^{-16}$**
Age at onset[b]	728	0.996	**0.024**	2110	0.998	0.065	2838	0.998 (0.996–0.999)	**0.013**
Disease course (PP)[c]	712	0.928	0.27	2106	1.031	0.42	2818	1.008 (0.944–1.076)	0.81
MSSS[c]	679	1.021	**0.0032**	1761	1.009	0.064	2440	1.014 (1.006–1.021)	**4.0×10^{-4}**
Disease duration lumbar puncture[d]	707	1.002	0.55	1899	0.998	0.35	2606	0.999 (0.996–1.002)	0.65
OCB-status versus	***n***	**OR[f]**	***P***	***n***	**OR[f]**	***P***	***n***	**OR OCB pos (95% CI)[f]**	***P***
Gender (female)[a]	3026	1.172	0.19	3842	1.406	**0.0011**	6868	1.298 (1.111–1.514)	**9.7×10^{-4}**
Age at onset[b]	2607	0.982	**0.0033**	3753	0.992	0.092	6360	0.989 (0.982–0.996)	**0.0028**
Disease course (PP)[c]	2549	1.066	0.74	3715	0.894	0.49	6264	0.989 (0.782–1.261)	0.93
MSSS[c]	1694	1.040	0.18	2401	1.005	0.85	4095	1.023 (0.987–1.060)	0.21
Disease duration lumbar puncture[d]	1343	1.012	0.39	2154	0.997	0.81	3497	1.003 (0.987–1.020)	0.71

PP = primary progressive; MSSS = Multiple Sclerosis Severity Score. Covariates included: [a]country; [b]country and gender; [c]country, gender and age at onset; [d]country, gender and age at lumbar puncture (LP). [e]Fold change in IgG index; [f]odds ratio for OCB positivity. Nominally significant *P*-values are indicated in bold.

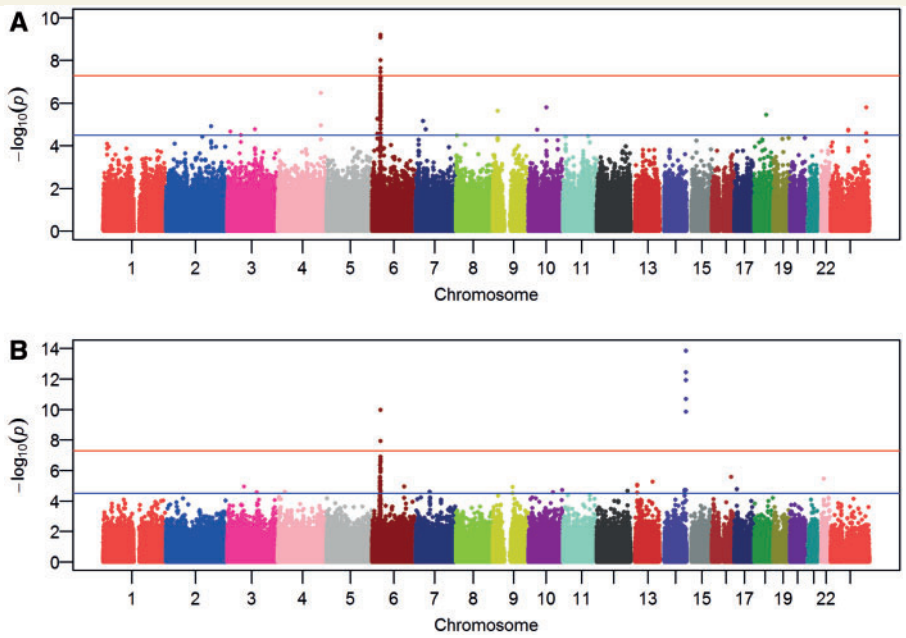

Figure 1 Manhattan plot of genome-wide association screen for (A) OCB status and (B) IgG index. Red line indicates genome-wide significance, blue line suggestive evidence.

contribution to the overall multiple sclerosis risk of each of these SNPs is small, we also estimated the multiple sclerosis genetic burden. This genetic risk score was composed of 57 established non-HLA multiple sclerosis risk SNPs and four classical HLA alleles (Gourraud *et al.*, 2011; The International Multiple Sclerosis Genetics Consortium and The Wellcome Trust Case Control Consortium 2, 2011). A higher multiple sclerosis genetic burden including the HLA alleles was associated with increased IgG index {fold change = 1.11 [95% confidence interval (CI): 1.07–1.15], $P = 1.33 \times 10^{-9}$} and OCB status [OR = 1.51 (95% CI: 1.32–1.74), $P = 3.44 \times 10^{-9}$], whereas these associations were substantially reduced when considering only non-HLA risk SNPs [IgG index: fold change = 1.06 (95% CI: 1.00–1.13), $P = 0.038$; OCB: OR = 1.19 (95% CI: 0.96–1.48), $P = 0.12$], indicating the MHC region as the main contributor.

Subsequently, we investigated SNPs known from the literature to be associated with the presence of antibodies in diverse other tissues (Pubmed and Catalogue of Genome-wide

Table 3 Genetic associations for OCB -positive versus -negative patients with multiple sclerosis

		Screen (n = 3026)				Replication (n = 3478)			Combined (n = 6504)	
CHR	POS	SNP	Minor Allele	P	OR	SNP	P	OR	P	OR (95% CI)
Primary signals										
2	179005067	rs3997876	A	1.22×10^{-5}	0.65	NA	NA	NA	NA	NA
3	12763938	rs1467025	A	2.20×10^{-5}	0.71	rs1467025	0.88	0.99	0.0030	0.85 (0.76–0.95)
3	53531263	rs979220	G	3.12×10^{-5}	0.69	rs979220	0.85	1.02	0.0057	0.84 (0.74–0.95)
3	109630418	rs3900940	G	1.74×10^{-5}	1.51	NA	NA	NA	NA	NA
4	169864539	rs9312352	G	3.35×10^{-7}	0.6	rs9312352	0.22	1.14	0.0040	0.81 (0.7–0.93)
6	19718096	rs9465463	A	5.54×10^{-6}	0.57	rs2743602[a]	0.67	1.06	NA	NA
6	32700178	rs9271640	A	6.21×10^{-10}	1.81	rs9271640	**2.15×10^{-4}**	1.38	**4.91×10^{-12}**	1.56 (1.38–1.77)
7	30113824	rs12701006	A	7.00×10^{-6}	0.55	rs12701006	0.89	0.98	6.37×10^{-4}	0.71 (0.58–0.86)
7	40906212	rs11767956	G	1.74×10^{-5}	1.44	rs11767956	0.94	0.99	0.0050	1.17 (1.05–1.31)
9	18243014	rs11790235	A	2.36×10^{-6}	0.48	rs11790235	0.22	0.83	2.44×10^{-5}	0.63 (0.51–0.78)
10	34249465	rs743107	G	1.78×10^{-5}	0.65	rs743107	0.5	0.94	4.80×10^{-4}	0.78 (0.68–0.9)
10	71371517	rs3793821	G	1.57×10^{-6}	0.61	rs16927103[b]	0.38	0.91	NA	NA
18	46778159	rs9807334	A	3.58×10^{-6}	0.58	rs9807334	**0.02**	0.76	**8.45×10^{-7}**	0.66 (0.56–0.78)
23	72267781	rs723923	A	1.78×10^{-5}	0.51	NA	NA	NA	NA	NA
23	142351548	rs6636962	A	1.59×10^{-6}	0.61	rs6636962	1.00	1.00	7.01×10^{-4}	0.78 (0.68–0.9)
Conditional signals										
3	108688432	rs1299704	G	7.14×10^{-5}	0.73	NA	NA	NA	NA	NA
6	32601567	rs34083746	G	4.14×10^{-5}	0.61	rs3957148[c]	0.059	0.80	2.38×10^{-4}	0.73 (0.62–0.86)
Previously reported associations										
3	141582383	rs17411949	A	0.0002	0.56	rs17411949	0.65	1.08	0.014	0.75 (0.6–0.94)
6	98125801	rs9320598	G	0.00298	0.63	rs9489141[d]	0.23	0.84	NA	NA

Correlation between replication and screen SNPs: [a]$r^2 = 1$; [b]$r^2 = 0.79$; [c]$r^2 = 0.63$; [d]$r^2 = 1$. CHR = chromosome; POS = position (hg18). Signals meeting criteria for replication are indicated in bold.

Table 4 Genetic associations for IgG index in patients with multiple sclerosis.

		Screen (n = 938)				Replication (n = 2072)			Combined (n = 3010)	
CHR	POS	SNP	Minor allele	P	Beta log IgG index	SNP	P	Beta log IgG index	P	Fold change IgG index (95% CI)
Primary signals										
3	65383112	rs12493497	C	1.16×10^{-5}	0.138	rs12493497	0.40	0.018	0.0015	1.04 (1.02–1.07)
3	115661169	rs10934275	G	2.68×10^{-5}	−0.179	rs10934275	0.35	−0.028	0.0014	0.95 (0.92–0.98)
4	26927863	rs4692195	G	2.52×10^{-5}	−0.151	rs4692195	0.73	−0.008	0.0080	0.96 (0.94–0.99)
6	32771829	rs6457617	A	1.07×10^{-10}	−0.206	rs6457617	**1.29×10^{-8}**	−0.127	**6.72×10^{-17}**	0.90 (0.88–0.92)
6	125378498	rs1413598	A	1.15×10^{-5}	0.151	NA	NA	NA	NA	NA
7	56016231	rs3816089	A	2.54×10^{-5}	0.264	rs3816089	0.91	0.005	0.012	1.06 (1.01–1.12)
9	77456395	rs1328565	G	1.22×10^{-5}	0.198	rs1328565	0.93	−0.003	0.018	1.04 (1.01–1.08)
10	95961456	rs12769135	C	2.72×10^{-5}	−0.148	rs12769135	0.32	0.024	0.13	0.98 (0.95–1.01)
10	132158923	rs448669	C	2.00×10^{-5}	0.186	rs448669	0.25	−0.033	0.17	1.02 (0.99–1.06)
12	120146349	rs3887080	A	2.24×10^{-5}	0.2	rs3887080	0.10	0.054	1.60×10^{-4}	1.07 (1.03–1.11)
13	26015959	rs10507368	G	8.63×10^{-6}	−0.163	rs10507368	0.93	0.002	0.020	0.97 (0.94–1.00)
13	86546361	rs9516451	A	5.55×10^{-6}	0.284	rs9516451	0.34	−0.040	0.086	1.04 (0.99–1.09)
14	99354933	rs4905899	A	1.92×10^{-5}	−0.134	rs4905899	0.35	−0.020	0.0015	0.96 (0.94–0.99)
14	105243925	rs11621145	G	1.48×10^{-14}	0.263	rs11621145	**1.97×10^{-24}**	0.23	**3.79×10^{-37}**	1.18 (1.13–1.22)
16	75867997	rs2343047	C	2.72×10^{-6}	−0.151	rs1594049[a]	0.23	−0.026	NA	NA
17	9982874	rs12162156	A	1.69×10^{-5}	0.15	rs12162156	0.54	0.014	0.0040	1.04 (1.01–1.07)
22	24768495	rs6004913	G	3.48×10^{-6}	0.183	rs6004913	0.93	0.002	0.0066	1.04 (1.01–1.08)
Conditional signals										
3	117229505	rs9289038	C	0.0001	−0.145	rs9289038	0.28	0.0337	0.10	0.97 (0.94–1.01)
6	30183822	rs3132679	A	6.61×10^{-5}	0.151	rs3132679	0.10	0.0444	2.60×10^{-4}	1.06 (1.03–1.09)
7	56540030	rs10249528	C	5.02×10^{-6}	0.158	rs10256440[b]	0.21	−0.0303	NA	NA

Correlation between replication and screen SNPs: [a]$r^2 = 0.97$; [b]$r^2 = 1$. Covariates: gender and seven principal components in screening phase, gender in replication phase. CHR = chromosome; POS = position (hg18). Signals meeting criteria for replication are indicated in bold.

Association Studies 4/12/2012) (Supplementary Table 4). A SNP tagging HLA-DRB1*15:01 (rs3129934), which is reported to be protective against IgA deficiency, increased IgG index ($P = 1.37 \times 10^{-7}$) as well as the risk of OCB positivity in multiple sclerosis ($P = 3.7 \times 10^{-4}$).

Replication

A total of 42 SNPs were taken forward to the replication phase and 38 SNPs were successfully genotyped (Supplementary Table 5). After quality control (Supplementary Table 6), 3478 patients with OCB status and 2072 with IgG index data available were analysed. The same two regions that were associated with genome-wide significance with IgG index in the screening phase, i.e. the MHC and IGHC region, reached genome-wide significance in the replication phase as well (Tables 3 and 4). One novel non-MHC region (rs9807334 near *ELAC1/SMAD4*) was replicated with nominal significance for association with OCB status.

Combined analyses

Given the highly significant correlation between OCB status and IgG index, we compared results from a detailed analysis of the MHC class II region for both characteristics (Supplementary Table 7 and Fig. 2). The most likely model in the combined analysis included two independent effects in the MHC class II region on OCB status; a major effect of the rs9271640*A-rs6457617*G haplotype and an additional effect of rs3957148 (tagging SNP with $r^2 = 0.63$ for rs34083746 identified in the screening phase). The first signal is highly correlated with the HLA-DRB1*1501 haplotype ($r^2 = 0.94$), whereas the second is strongly correlated with HLA-DQA1*0301 (rs34083746: $r^2 = 0.93$, rs3957148: $r^2 = 0.64$), and more modestly with both HLA-DRB1*0401 (rs34083746: $r^2 = 0.39$) and *0404 (rs34083746: $r^2 = 0.26$). Carrying the MHC SNP allele associated with OCB-positive status at these loci, was associated with a 2-fold higher likelihood of being OCB-positive than carrying the allele associated with OCB-negative status at all three loci [OR = 2.25 (95% CI: 1.84–2.75), $P = 8.88 \times 10^{-16}$] (Fig. 2A). The rs9271640*A-rs6457617*G haplotype, correlated with DRB1*1501, was associated not only with OCB status, but also IgG index ($P = 1.59 \times 10^{-22}$) (Fig. 2B). Independent of this haplotype, rs6457617*G had an additional effect increasing IgG index ($P = 3.68 \times 10^{-6}$). Notably, the association of rs6457617 with IgG index was observed in both OCB-positive ($P = 4.22 \times 10^{-10}$) and OCB-negative multiple sclerosis patients ($P = 6.12 \times 10^{-4}$). SNP rs6457617 is only modestly associated with any classical HLA allele ($r^2 < 0.30$). SNP rs34083746/rs3957148, correlated with DQA1*0301 and associated with OCB negative status, did not seem to influence IgG index levels. No interaction amongst the associated SNPs in the MHC region or between these and the SNP in the IGHC region was observed (data not shown).

The previously reported association of IgG index with SNPs in the IGHC region was replicated as highly significant in the combined data set ($P = 3.79 \times 10^{-37}$) and explains 4.7% of the variance in IgG index in the entire data set. The MHC and IGHC region, the major determinants of variation in IgG index, together explain 7.75% of variance and combined with gender and country, account for 12.65% of variance in IgG index throughout Europe. Forest plots for all association signals per country are given in Supplementary Figs 5 and 6.

We also investigated the correlation between the main four established genetic effects and age at onset, disease course and Multiple Sclerosis Severity Score with covariates gender and, for the latter two, age at onset. After correcting for multiple testing, the A-allele of rs9271640 (that was associated with increased IgG levels and likelihood of OCB-positive status), was associated with lower age at onset ($P_{screen} = 4.44 \times 10^{-4}$, $P_{replication} = 0.082$, $P_{combined} = 5.68 \times 10^{-4}$). In contrast, at the IGHC locus, the allele that increased IgG index showed association with a later age at onset ($P_{screen} = 0.011$, $P_{replication} = 0.0066$, $P_{combined} = 8.23 \times 10^{-5}$) (Supplementary Fig. 7).

Discussion

This study is the largest to date that investigates genetic differences, clinical and demographic characteristics in relation to OCB status ($n = 6868$) and IgG index ($n = 3126$) in multiple sclerosis. Our findings strongly support that multiple sclerosis patients with and without OCBs and/or increased IgG index are genetically distinct. This has previously been suggested in other, smaller studies (Kikuchi *et al.*, 2003; Imrell *et al.*, 2006; Idiman *et al.*, 2009; Wu *et al.*, 2009; Romero-Pinel *et al.*, 2011; Buck *et al.*, 2012; Leone *et al.*, 2013; Mero *et al.*, 2013; Yoshimura *et al.*, 2014) and is now confirmed. In accordance with our previous observations and those of others (Lechner-Scott *et al.*, 2011; Mero *et al.*, 2013; Stangel *et al.*, 2013), patients with multiple sclerosis and high CSF antibody levels, as characterized by OCB-positive status and/or high IgG index, more often are female and seem to have a lower age at onset and higher Multiple Sclerosis Severity Score. We find that the two CSF parameters included in this study, OCB status and IgG index, are highly correlated (Mayringer *et al.*, 2005; Link and Huang, 2006; Rinker *et al.*, 2007), supporting that these measurements reflect the same immunological process.

Recently, the IGHC locus on chromosome 14 was reported to be associated with IgG index in German and Belgian patients with multiple sclerosis (Buck *et al.*, 2012). This is convincingly replicated in the present extended study with the finding of a strong association of rs11621145 to IgG index ($P_{combined} = 3.79 \times 10^{-37}$). In addition, we identify two novel association signals in the MHC region as major genetic determinants for IgG index; rs9271640, which is correlated with the HLA-DRB1*15:01

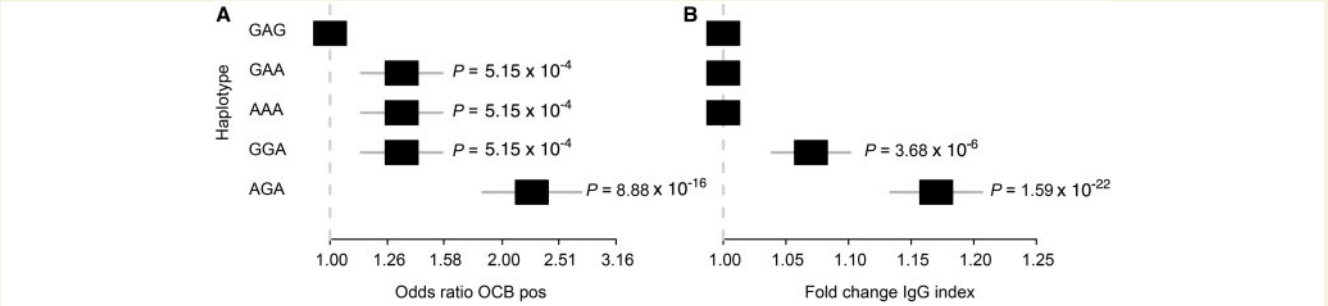

Figure 2 Most likely model for MHC class II haplotypes (composed of SNPs rs9271640-rs6457617-rs3957148) and CSF phenotype in the combined analysis. (**A**) OCB status and (**B**) IgG index. GAG haplotype is correlated with HLA-DQA1*0301 ($r^2 = 0.64$), AGA haplotype with HLA-DRB1*1501 ($r^2 = 0.94$). *P*-values are given compared to reference haplotypes.

allele, and rs6457617, which is located near the HLA-DQA1 gene but not known to tag any conventional HLA-allele. An interaction between HLA and IGHC has been suggested (Pandey, 2013); however, we do not find any evidence to support this hypothesis. Together, these three SNPs from the MHC and IGHC regions explain ~7.75% of the variance in IgG index.

Several studies have suggested that OCB-positive and -negative patients with multiple sclerosis are associated with different HLA-DRB1 alleles (Kikuchi *et al.*, 2003; Imrell *et al.*, 2006; Idiman *et al.*, 2009; Wu *et al.*, 2009; Romero-Pinel *et al.*, 2011; Leone *et al.*, 2013; Mero *et al.*, 2013; Yoshimura *et al.*, 2014). We observe a significant association of SNP rs9271640 tagging HLA-DRB1*15:01 with OCB status and a high correlation of the multiple sclerosis genetic burden including HLA risk alleles with OCB status. Our findings hence support previous observations of the major established multiple sclerosis risk allele, HLA-DRB1*15:01, being more strongly associated with OCB-positive multiple sclerosis than OCB-negative multiple sclerosis. The present study also detects a significant difference between OCB-positive and -negative patients with regard to rs34083746. This SNP is highly correlated with the DQA1*0301 allele shared between DRB1*04 haplotypes. Hence, our findings are consistent with several HLA-DRB1*04 alleles having been shown to increase risk for OCB-negative multiple sclerosis, but not OCB-positive multiple sclerosis, in previous studies from Europe and Japan (Kikuchi *et al.*, 2003; Imrell *et al.*, 2006; Mero *et al.*, 2013; Yoshimura *et al.*, 2014). The combination of variation in the MHC region has a major impact on the OCB status of patients with multiple sclerosis, resulting in a >2-fold difference in the odds of being OCB-positive. In addition to replicating previously suggested associations to OCB status within the MHC region, our analyses provide evidence for a novel locus to be associated with OCB status, rs9807334 near the *ELAC1/SMAD4* genes. The *SMAD4* gene, a signal transduction protein in the tumor growth factor beta pathway, has previously been implicated in class switch recombination and in experimental autoimmune encephalomyelitis and multiple sclerosis (Park *et al.*, 2005; Meoli *et al.*, 2010; Huss *et al.*, 2011) and the same allele has previously been suggested as associated with vaccine response (Ovsyannikova *et al.*, 2012).

The CSF phenotype association signals in the MHC region we observe have been associated with susceptibility and antibody levels in other diseases. The HLA-DRB1*1501-DQB1*0601 haplotype has been associated with either the presence or increased quantity of immunoglobulins of the IgG, IgA and IgM families both in healthy controls and in disease, including total immunoglobulins (Ferreira *et al.*, 2010), antibodies induced by viruses such as Epstein-Barr virus (Rubicz *et al.*, 2013), and autoantibodies in type 1 diabetes (Ishii *et al.*, 2005) and Sjögren syndrome (Gottenberg *et al.*, 2003) though an opposite correlation is seen for few other antibody responses (Sundqvist *et al.*, 2014). HLA-DQA1*0301, highly correlated with SNP rs34083746, is associated with autoantibody negative disease in ketosis-prone diabetes (Oak *et al.*, 2014). SNP rs6457617 is a major susceptibility factor in rheumatoid arthritis and systemic sclerosis, independent of classical HLA alleles (The International MHC and Autoimmunity Genetics Network, 2009; Radstake *et al.*, 2010; Allanore *et al.*, 2011; Orozco *et al.*, 2014). Of note, the risk allele for these autoimmune diseases decreases the IgG levels in multiple sclerosis. The IGHC locus, on the other hand, has not been associated with antibody levels in other diseases as yet. Overall, the MHC region may enhance antibody production and OCB-positive status, whereas the IGHC region has been proposed to influence clearance of antibodies from the CSF and hence immunoglobulin levels (Buck *et al.*, 2012).

Other studies have shown that IgG levels correlate with the number of B cells, more particularly the number of plasmablasts, in the CSF (Rudick *et al.*, 1999; Cepok *et al.*, 2001, 2005). B cell follicles in the meninges have been found in a subset of patients with multiple sclerosis and are suggested to play a pathogenic role (Magliozzi *et al.*, 2007). Moreover, a prognostic potential of findings in the CSF has been proposed, as positivity for OCB is reported to

double the risk of developing clinically definite multiple sclerosis in patients with clinically isolated syndrome (Tintore *et al.*, 2008). Also, some studies suggest that the inflammation in the brain reflected by CSF alterations may correlate with neurodegeneration and disease progression (Stangel *et al.*, 2013). Our findings of a lower age at onset and a higher Multiple Sclerosis Severity Score in the patients with multiple sclerosis with marked CSF antibody levels support this hypothesis. In our study, HLA-DRB1*15:01 increases CSF antibody levels and lowers age at onset, as reported previously (Hensiek *et al.*, 2002; The International Multiple Sclerosis Genetics Consortium and The Wellcome Trust Case Control Consortium 2, 2011). However, at the IGHC locus, the allele increasing IgG index seems correlated with higher age at onset, indicating that the mechanisms underlying the correlation between genetic determinants, CSF antibody levels and clinical outcome are not fully understood as yet.

The present study is well-powered, and the clinical characteristics of the patients included support that they are representative of patients with multiple sclerosis in general. On average, OCB positivity was found in 88% and an elevated IgG index in 64% of patients, in accordance with earlier studies (Dobson *et al.*, 2013; Stangel *et al.*, 2013). Lumbar puncture is routinely performed as a part of the diagnostic process in most of the countries included, but a possible selection bias must be kept in mind when interpreting the clinical findings. OCBs and/or increased IgG index in the CSF are clinically important hallmarks in multiple sclerosis, and in this large study we show that these disease phenotypes are associated with both genetic variants and clinical and demographic characteristics. The presence of intrathecal immunoglobulin M has been reported as an additional CSF characteristic in multiple sclerosis patients that may be associated with unfavourable outcome or aggressive disease (Stangel *et al.*, 2013). The study of genetic factors underlying immunoglobulin M levels is currently hampered by the availability of data but will be of future interest.

In summary, in this large study including 6950 patients with multiple sclerosis, we confirm that genetic variants in the immunologically important regions of MHC and IGHC influence OCB status and IgG index in patients with multiple sclerosis.

Acknowledgements

We thank the patients who participated in this study. We acknowledge the International Multiple Sclerosis Genetics Consortium (IMSGC) and Wellcome Trust Case Control Consortium 2 (WTCCC2) for the collaboration with the establishment of the genotypes that were included in the screening phase of this project. Please see Supplementary material for the IMSGC membership list. We thank Leentje Cosemans and the Genomics Core Facility at the KU Leuven (Belgium) for technical assistance with the replication phase and Prof. Koen Poesen for advice. From Norway we thank collaborators from the Multiple Sclerosis Research group in Oslo, especially Anja Bjølgerud and Inger-Lise Mero, and the Norwegian Multiple Sclerosis Register and Biobank for contribution with samples and data. The Italian group thanks the PROGEMUS (PROgnostic GEnetic factors in MUltiple Sclerosis) Consortium, the PROGRESSO Consortium, Dr Mariaemma Rodegher, Dr Lucia Moiola, Dr Bruno Colombo, Dr Vittorio Martinelli, Dr ssa Gabriella Passerini and Prof. Giancarlo Comi. The German group gratefully acknowledges Verena Grummel for her continuous contributions to the DNA biobank. From Australia, Prof. William Carroll, Dr Marzena Fabis-Pedrini, Mr Sean Burnard and Mrs Malgorzata Krupa are thanked for their contributions.

Funding

The Belgian contribution to the project was supported by the Research Fund KU Leuven (OT/11/087 to A.G. and B.D.), the Belgian Charcot Foundation (Research Grant to A.G.), and the Research Foundation Flanders (FWO-Vlaanderen; Clinical Investigator Fellowship to B.D.). The Norwegian part of the study was supported by The Research Council of Norway (project 143153 and 196776 by H.F.H.), University of Oslo (project 211567 by H.F.H.), NevroNor, Research Council of Norway (project 176958 by K.M.M.) and The Western Norway Regional Health Authority, Stavanger, Norway. The Italian contribution was supported by the Italian Foundation for Multiple Sclerosis [FISM grants 'Progetto Speciale Immunochip', 2011/R/14, Fondazione cariplo (n° 2010-0728)], and the CRT Foundation, Turin, Italy. The German contribution was supported by the German Ministry for Education and Research (BMBF), the German Competence Network Multiple Sclerosis (KKNMS, Control-MS, 01GI0917), the Deutsche Forschungsgemeinschaft (SyNergy Cluster, JE 530/1-1), and the Munich Biomed Cluster M4. The Danish contribution was supported by grants from the Danish Multiple Sclerosis Society, the Danish Council for Strategic Research [grant number 2142-08-0039] and the Warwara Larsen Foundation. The Spanish contribution was supported by the 'Red Española de Esclerosis Múltiple (REEM)' sponsored by the FEDER-FIS and the 'Ajuts per donar Suport als Grups de Recerca de Catalunya', sponsored by the 'Agència de Gestió d'Ajuts Universitaris i de Recerca' (AGAUR), Generalitat de Catalunya, Spain. P.A.G. is a recipient of the Race to erase MS Award and the European Federation for Immunogenetics Julia Bodmer Award.

Supplementary material

Supplementary material is available at *Brain* online.

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