Genome-wide Scan of 500,000 Single-Nucleotide Polymorphisms Among Responders and Nonresponders to Interferon Beta Therapy in Multiple Sclerosis

Manuel Comabella, MD; David W. Craig, BSc; Carlos Morcillo-Suárez, BSc; Jordi Río, MD; Arcadi Navarro, PhD; Marta Fernández, BSc; Roland Martin, MD; Xavier Montalban, MD, PhD

Background: Interferon beta is 1 of 2 first-line treatments for relapsing-remitting multiple sclerosis (MS). However, not all patients respond to interferon beta therapy, and to date there is a lack of surrogate markers that reliably correlate with responsiveness to interferon beta therapy in MS.

Objective: To identify allelic variants that influence response to interferon beta therapy in patients with MS.

Design: Genome-wide scan.

Setting: Academic research.

Patients: Two hundred patients having relapsing-remitting MS treated with interferon beta and having a follow-up period of at least 2 years were classified as responders or nonresponders to treatment based on stringent clinical criteria.

Main Outcome Measures: In the first phase of the study, a pooling-based genome-wide association study of 428,867 single-nucleotide polymorphisms (SNPs) was performed in 53 responders and 53 nonresponders to interferon beta therapy. After applying several selection criteria, 383 SNPs were individually genotyped in an independent validation cohort of 49 responders and 45 nonresponders to interferon beta therapy using a different genotyping platform.

Results: Eighteen SNPs had uncorrected P<.05 associated with interferon beta responder status in the validation cohort. Of these, 7 SNPs were located in genes that code for alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid–type glutamate receptor GRIA3, type 1 interferon–related proteins ADAR and IFNAR2, cell cycle–dependent protein CIT, zinc finger proteins ZFAT and ZFHX4, and guanosine triphosphatase–activating protein STARD13.

Conclusions: This study supports an underlying polygenic response to interferon beta treatment in MS and highlights the importance of the glutamatergic system in patient response to interferon beta therapy.


ECCOMBINANT HUMAN INTERFERON beta therapy is a mainstay of multiple sclerosis (MS) treatment and has demonstrated a beneficial effect on disease activity, as measured by clinical and magnetic resonance imaging variables.1-3 However, interferon beta therapy is only partially effective, and 20% to 55% of treated patients will show a lack of response to treatment.4 Unfortunately, clinical and radiologic criteria to classify patients as interferon beta therapy responders or nonresponders are usually applied late, after 1 or 2 years of follow-up. To improve the efficacy of treatments for MS, it is desirable to find biomarkers that allow early identification of treatment responders or ideally that predict responder status.

Several pharmacogenetic studies5-11 aimed to identify allelic variants that influence response to interferon beta therapy in MS. In these studies, interferon beta–regulated genes or HLA class II alleles were proposed as potential biomarkers for treatment response. Unfortunately, results from these studies revealed no association or weak and unreplicated associations of candidate genes with response to interferon beta therapy. To date, only one genome-wide pharmacogenetic study12 has been published in the field of MS. The study identified candidates with response to interferon beta treatment and found an overrepresentation of genes related to ion chan-

Author Affiliations are listed at the end of this article.
nerves and signal transduction pathways such as γ-amino-
butyric acid or glutamate receptor genes.

Herein, we present the results of a genome-wide scan. Our pharmacogenetic study was performed using pooled DNA and single-nucleotide polymorphism (SNP) genotyping arrays.

**Table 1. Demographic and Clinical Characteristics of Responders and Nonresponders to Interferon Beta Therapy**

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Responders (n=53)</th>
<th>Nonresponders (n=53)</th>
<th>P Value</th>
<th>Responders (n=49)</th>
<th>Nonresponders (n=45)</th>
<th>P Value</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>32.5 (8.5)</td>
<td>35.5 (8.5)</td>
<td>.23</td>
<td>34.5 (8.0)</td>
<td>35.0 (9.7)</td>
<td>.79</td>
<td>.87</td>
</tr>
<tr>
<td>Female to male ratio (% female)</td>
<td>37:16 (69.8)</td>
<td>37:16 (69.8)</td>
<td>.99</td>
<td>33:16 (67.3)</td>
<td>37:8 (82.2)</td>
<td>.10</td>
<td>.46</td>
</tr>
<tr>
<td>Duration of disease, mean (SD), y</td>
<td>6.2 (6.3)</td>
<td>7.1 (5.6)</td>
<td>.47</td>
<td>5.5 (5.1)</td>
<td>5.4 (5.3)</td>
<td>.94</td>
<td>.15</td>
</tr>
<tr>
<td>EDSS score, mean (IQR)</td>
<td>2.1 (1.0-2.8)</td>
<td>2.8 (1.5-3.5)</td>
<td>.01</td>
<td>2.2 (1.5-2.5)</td>
<td>2.6 (2.0-3.5)</td>
<td>.047</td>
<td>.84</td>
</tr>
<tr>
<td>No. of relapses, mean (SD)</td>
<td>2.6 (1.0)</td>
<td>2.9 (1.4)</td>
<td>.21</td>
<td>2.9 (1.0)</td>
<td>2.6 (2.2)</td>
<td>.41</td>
<td>.94</td>
</tr>
</tbody>
</table>

**METHODS**

**STUDY DESIGN AND CLINICAL ASSESSMENT**

We studied patients having relapsing-remitting MS (RRMS) treated with interferon beta at the outpatient clinic of the Centre d’Esclerosi Múltiple de Catalunya, Barcelona, Spain. All patients were included in a follow-up protocol that collected demographic and basal and longitudinal clinical data, including number of relapses and Expanded Disability Status Scale (EDSS) scores, as previously described.1 The study was approved by the local ethics committee, and all patients gave their informed consent.

**DEFINITION OF RESPONSE TO INTERFERON BETA THERAPY**

Clinical criteria of response to interferon beta therapy were applied after 2 years of treatment. Patients were considered responders if there was no increase in the EDSS score and no relapses during the follow-up period. Patients were considered nonresponders if during the follow-up period there was 1 or more relapses and an increase of at least 1 point in the EDSS score that persisted for at least 2 consecutive scheduled visits separated by a 6-month interval.1

**ORIGINAL COHORT**

One hundred six patients with RRMS, 53 (50.0%) responders and 53 (50.0%) nonresponders, were included in the study. Demographic and baseline clinical characteristics of the patients are summarized in Table 1.

**SAMPLE POOLING AND HIGH-DENSITY SNP GENOTYPING**

DNA samples were checked for quality using 2% agarose gel electrophoresis, and degraded samples were excluded from the pooling analysis. The genomic DNA concentration of each subject was determined in triplicate using a kit (Quant-iT PicoGreen dsDNA Assay Kit; Invitrogen, Carlsbad, California) according to the manufacturer’s recommendations. These triplicate values were used to calculate a median concentration for each DNA sample. Individual DNA samples were then added to their respective pools in equivalent molar amounts. Once created, each pool was diluted to 50 ng/µL with sterile water in preparation for genotyping. DNA samples from responders and nonresponders were pooled separately. All samples were pooled 3 times to account for pipetting variability. Each of these replicates were genotyped on 3 replicate arrays to account for assay and array variability. Nine early access arrays (300K; Affymetrix, Inc, Santa Clara, California) were used, following the protocols of the manufacturer (http://www.affymetrix.com).

**ANALYSIS OF POOLED DATA FROM THE SNP ARRAYS**

Probe intensity data were directly read from cell intensity files, and relative allele signal values were calculated for each quartet. Relative allele signal values correspond to the ratio of the A probe to the sum of the A and B probes (in which A is the major allele and B is the minor allele) and provide a quantitative index correlating to allele frequencies in pooled DNA. These values yield independent measures of different hybridization events and are consequently treated as individual data points. Quality control was assessed by determination of the correlation coefficient of the relative allele signal values across replicates, followed by unsupervised hierarchical clustering. Quantitatively, Pearson product moment correlation coefficients were also used, and all arrays exceeded a correlation coefficient of 0.95. In this experiment, no poor-performing arrays were identified. Although not performed in this study, we advise using intraclass correlations between arrays because they provide a better indication of agreement between arrays for quality control. Analysis was completed in an identical fashion to several
other investigations using pooled genomic DNA, and further description is available elsewhere. A silhouette statistic, which represents the mean of the distance of a point to all other points in its class (ie, responder pool) vs points in the other class (ie, nonresponder pool), was used to rank all genotyped SNPs. This test statistic has been heuristically shown to perform well in identifying SNPs with large allelic frequency differences.

**GENE ONTOLOGIC ANALYSIS**

Gene ontologic enrichment analysis was performed in the top 2% of SNPs using commercially available software (Metacore; GeneGo, Inc, St Joseph, Michigan), whereby genes were tested for preferential membership in 1 of several predefined pathways. Statistical significance was obtained from the manufacturer's suite.

**VALIDATION OF TOP CANDIDATE SNPs**

**BY INDIVIDUAL GENOTYPING**

We applied 2 criteria to select candidate SNPs for validation in an independent cohort of responders and nonresponders. A first criterion was to select the top 100 high-scoring SNPs from the arrays obtained after ranking SNPs using a silhouette statistic, as described in the preceding subsection. A second criterion was to identify clusters of 2 or more SNPs located in close proximity and scoring in the top 2% and subsequently to select from each cluster the SNPs having the highest scores.

Three hundred eighty-three SNPs were chosen for individual genotyping in a validation cohort comprising 94 patients with interferon beta–treated RRMS who attended the outpatient clinic of the Centre d’Esclerosi Multiple de Catalunya and were classified as responders or nonresponders to therapy based on the same stringent clinical criteria as those used in the original pooled cohort. There were 49 responders (52.1%) and 45 nonresponders (47.9%) to therapy. None of these patients were part of the original cohort used for DNA pooling. Demographic and clinical characteristics of responders and nonresponders are summarized in Table 1. Except for EDSS score, responders and nonresponders were comparable on all cohort variables. The baseline EDSS score was higher in nonresponders compared with responders, a finding that has been reported previously. Comparisons between cohorts revealed a higher percentage of patients treated with interferon beta-1b (subcutaneous administration) and a lower percentage of patients treated with interferon beta-la (intramuscular administration) in the original cohort compared with the validation cohort.

DNA samples from the validation cohort were quantitated at the National Genotyping Center (Madrid, Spain) using the previously described assay (Quant-iT PicoGreen dsDNA Assay Kit). Another assay (GoldenGate; Illumina Inc, San Diego, California) was used to individually genotype SNPs selected from the platform (Affymetrix 500K). Seventeen SNPs were discarded because of problems in the genotyping process. One SNP was discarded because of significant departure from Hardy-Weinberg equilibrium. Statistical significance of individual genotype data was calculated as an allelic χ² value. This statistical model was chosen because it is analogous to the type of allelic associations that would be detectable using a pooling-based genome-wide association study. Quality control processes and allelic and genotypic association tests were performed using software available on the Internet (SNPator [http://www.snpator.com]).

**RESULTS**

We found an association between the X-linked GRIA3 gene (OMIM 305915), an alpha-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA)–type glutamate receptor, and response to interferon beta therapy. Response to treatment was also associated with polymorphisms located in type 1 interferon–related genes. Overall, the study findings reflect the complex and polygenic nature underlying response to interferon beta therapy in MS and underscore the importance of the glutamatergic system in response to treatment. The details of our findings follow.

**GENOTYPING OF THE ORIGINAL POOLED COHORT USING SNP ARRAYS**

A flowchart summarizing the main steps of the study design and analysis is shown in the Figure. In a first phase of the study, pooled DNA samples from 106 patients with RRMS classified as responders or nonresponders to interferon beta therapy based on stringent clinical criteria were hybridized to genotyping arrays (Affymetrix 500K). A ranked list of 428,867 SNPs was obtained from analysis of the probe intensity differences between pools of 53 responders and 53 nonresponders.

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**Figure.** Flowchart showing the phases in the analysis of the study data. With the use of genotyping arrays (500K; Affymetrix, Inc, Santa Clara, California), a ranked list of single-nucleotide polymorphisms (SNPs) was obtained on pooled DNA from 53 responders and 53 nonresponders to interferon beta (IFNβ) therapy classified on the basis of stringent clinical criteria such as score progression on the Expanded Disability Status Scale (EDSS) and number of relapses (original cohort). Gene ontologic enrichment analysis was performed in the top-scoring SNPs. After applying criteria based on SNP ranking and clustering, 383 SNPs were selected for individual genotyping in an independent cohort of 49 responders and 45 nonresponders to treatment (validation cohort) using a genotyping assay (GoldenGate; Illumina Inc, San Diego, California). Finally, association statistical tests and SNP interaction analyses were performed on the selected SNPs.
GENE ONTOLOGICAL ANALYSIS OF TOP SNPs

Functional data mining by gene ontologic analysis revealed that the immune response category associated with the antiviral actions of interferons was most represented in the top-ranked SNPs that best discriminated between responders and nonresponders. As summarized in Table 2, representative genes from this category are ADAR (OMIM 146920), IFNAR2 (OMIM 602376), NOS2 (OMIM 163730), and OAS3 (OMIM 603351). Two other less-represented categories among top-scoring SNPs were the interleukin 6 signaling and Ras-like protein A regulation pathways.

INDIVIDUAL GENOTYPING OF SELECTED SNPS IN A VALIDATION COHORT

In a second phase of the study, from the ranked list of SNPs and after applying the 2 selection criteria described in the “Methods” section, 383 SNPs were chosen for genotyping in a replication cohort to validate results from the pooled SNP arrays. We did not perform genotyping in our original pooled cohort because the objective of the study was not to evaluate pooling. Instead, we genotyped selected SNPs in individual DNA samples from a totally independent cohort of 94 interferon beta therapy responders and nonresponders by using a different genotyping platform. Calculated association statistics are given in Table 3 for the intragenic SNPs showing statistical significance ($P < .05$ after allelic frequency comparisons).

The SNP rs12557782 showed the greatest association with response to interferon beta therapy, with $P = .002$ and an odds ratio of 2.7 (Table 3). This SNP resides in chromosome X and is positioned in intron 2 of the GRIA3 gene, which codes for an AMPA-type glutamate receptor. At the genotype level, this X-linked gene showed different behavior between the sexes; a positive association with response to treatment was observed among women ($P < .001$; odds ratio, 8.5) but not among men ($P = .56$; odds ratio, 1.7). It cannot be ruled out that the validation failure in men is not due to power because fewer men were included in the study and men contribute only 1 copy of the X chromosome per individual. The second strongest association signal, with $P = .006$ and an odds ratio of 2.4, was found for SNP rs7308076, which is located in the C1IT gene (OMIM 605629) and codes for a cell cycle-dependent protein. Two SNPs associated with response to treatment reside in genes that are directly associated with the type 1 interferon pathway. One is SNP rs2229857, which is located in the RNA-specific adenosine deaminase gene ADAR gene and encodes an interferon-inducible protein with antiviral functions; this is a non-synonymous SNP that results in an amino acid change (arginine to lysine) in exon 2 of ADAR. The other is rs2248202, an intronic SNP positioned in IFNAR2, a gene that encodes 1 of 2 subunits that compose the type 1 interferon receptor heterodimer.

Three others SNPs were associated with treatment response (Table 3). These include rs733254 and rs11787532 (located in genes that code for the zinc finger proteins ZFAT and ZFHX4, respectively) and rs9527281 (positioned in the STARD13 gene (OMIM 609866), which encodes a Rho family guanosine triphosphatase-activating protein).

The following intergenic SNPs were also associated with response to treatment in comparisons of allelic frequencies between interferon beta therapy responders and nonresponders: rs4425528 (chromosome 5, $P = .009$), rs1501173 (chromosome 4, $P = .02$), rs10424648 (chromosome 19, $P = .03$), rs274947 (chromosome 9, $P = .03$), rs8180631 (chromosome 6, $P = .03$), rs7517690 (chromosome 1, $P = .03$), rs1948577 (chromosome 16, $P = .03$), rs11956421 (chromosome 5, $P = .03$), rs1382673 (chromosome 12, $P = .04$), rs236855 (chromosome 6, $P = .04$), and rs9297235 (chromosome 8, $P = .04$). The remaining SNPs were unassociated with responder status. The full list of SNPs is given in the eTable (http://www.archneurol.com).

INTERACTION ANALYSIS BETWEEN SNPS

Finally, interaction analysis between the selected SNPs used for validation was performed to evaluate whether combinations of SNPs increased the strength of association with response to treatment. No statistically significant interactions were obtained in combinations of pairs or triplets of SNPs. The strongest pair and triplet interactions selected by the software program (MDR 1.2.5) were pair rs417812-rs12557782 (testing balanced accuracy, 0.56; $P = .72$) and triplet rs2322668-rs417812-rs12557782 (testing balanced accuracy, 0.49; $P = .97$) ($\chi^2$ test for both).

<table>
<thead>
<tr>
<th>Category</th>
<th>Cell Process</th>
<th>Genes</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immune response–antiviral actions of interferons</td>
<td>Immune response</td>
<td>ADAR, IFNAR2, NOS2, OAS3</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Immune response–interleukin 6 signaling pathway</td>
<td>Cytokine- and chemokine-mediated signaling pathway, immune response</td>
<td>ADAM10, STAT3</td>
<td>.03</td>
</tr>
<tr>
<td>Cytoskeleton remodeling–RasA regulation pathway</td>
<td>Small guanosine triphosphatase–mediated signal transduction</td>
<td>CDC42, PGGT1B</td>
<td>.03</td>
</tr>
</tbody>
</table>

Table 2. Gene Ontologic Categories Overrepresented in the Top 2% of SNPs Obtained From the Arrays With Pooled DNA Samples

Abbreviations: Ras-like protein A; SNP, single-nucleotide polymorphism.

The growing number of new therapeutic strategies for MS and their potential risk for lack of response at the individual patient level make personalized therapy a ne-
cessity for MS. Pharmacogenomics is a new field that incorporates genome technologies such as gene expression profiling, SNP screens, and proteomics to predict treatment response. Although pharmacogenomics holds great promise for individualized therapy, it is still in its infancy in the field of MS.

Several technologies have been used in an attempt to identify genes associated with response to interferon beta therapy, and several surrogate markers for interferon beta treatment response have been proposed. Unfortunately, results from these studies were disappointing: many genetic associations with treatment response that were identified in some investigations were not confirmed in others. To date, only one genome-wide pharmacogenomic study on interferon beta therapy in MS has been published. This study, in which the Centre d’Esclerosi Multiple de Catalunya was one of 4 collaborating centers, aimed to identify genes associated with response to interferon beta therapy and was performed among responder and nonresponder DNA pools using genotyping arrays (Affymetrix 100K). Although previously reported genetic associations were not confirmed, the study identified candidate genes and underscored the genetic heterogeneity underlying response to interferon beta therapy in MS.

Herein, we also conducted a genome-wide association study with pooled DNA samples from responders and nonresponders to interferon beta therapy but used higher-density SNP arrays (Affymetrix 500K) and more stringent clinical criteria to identify treatment failure. The study was conducted in 2 phases. In the first phase of the study, a ranked list of SNPs was obtained from the analysis of the SNP microarrays. In the second phase of the study, top-scoring SNPs were individually genotyped in an independent replication cohort using a different genotyping platform. It is well known in replicated association studies that most SNPs do not show association in the replication cohort. In our study, only 18 of 383 SNPs selected from the microarrays showed statistical significance in the validation cohort. None of the reported allelic and genotypic associations exceeded multiple test correction. Nevertheless, lack of correction for multiple testing would have resulted in a high risk for false-positive results.

The strongest association signal corresponded to SNP rs12557782, a polymorphism located in the GRIA3 gene, which encodes an AMPA-type glutamate receptor. This finding is relevant from the pathogenetic point of view. A potential connection between genes that encode neurotransmitter-gated channels and response to interferon beta therapy was suggested by Byun and colleagues based on their findings of an overrepresentation of genes that code for glutamate and γ-aminobutyric acid receptors in gene ontologic analysis. Our results support the relationship between neuronal excitation and interferon beta treatment response. Glutamate has important roles in the central nervous system, and AMPA-type glutamate receptors mediate excitatory synaptic transmission in the central nervous system and are expressed in oligodendrocytes. Overactivation of AMPA receptors leads to excitotoxic oligodendroglial cell death and may be involved in the pathogenesis of demyelinating disorders. Although the mechanism is unknown whereby allelic variants of the GRIA3 gene may influence response to interferon beta therapy, this finding together with the observation that genetic association was restricted to women represents a new range of perspectives in the field of interferon beta pharmacogenomics.

### Table 3. Association Analysis of Statistically Significant SNPs After Genotyping in the Validation Cohort

<table>
<thead>
<tr>
<th>SNP</th>
<th>Chromosome Location</th>
<th>Gene</th>
<th>Location</th>
<th>Allelic Association</th>
<th>Genotypic Association</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs12557782</td>
<td>X</td>
<td>Glutamate receptor, ionotropic, AMPA 3 (GRIA3)</td>
<td>Intron 2</td>
<td>G 2.7 (1.5-5.2) .002</td>
<td>AG + GG in women, G in men 8.5 (2.2-32.8) .001</td>
</tr>
<tr>
<td>rs7308076</td>
<td>12</td>
<td>Citron (Rho-interacting, serine-threonine kinase 21) (CIT)</td>
<td>Intron 9</td>
<td>C 2.4 (1.3-4.4) .006</td>
<td>CC 3.7 (1.5-8.6) .003</td>
</tr>
<tr>
<td>rs2229857</td>
<td>1</td>
<td>Adenosine deaminase, RNA-specific (ADAR)</td>
<td>Exon 2 (missense)</td>
<td>A 2.1 (1.1-4.0) .02 AA</td>
<td>GT + 8.6 (1.0-71.7) .02</td>
</tr>
<tr>
<td>rs733254</td>
<td>8</td>
<td>Zinc finger and autoimmune thyroid hook domain-containing (ZFAT)</td>
<td>Intron 3</td>
<td>G 2.1 (1.1-4.0) .02</td>
<td>GG + GT 22.4 (1.3-401.2) .002</td>
</tr>
<tr>
<td>rs9527281</td>
<td>13</td>
<td>Steroidogenic acute regulatory gene-related lipid transfer domain-containing 13 (STARD13)</td>
<td>Intron 1</td>
<td>T 2.0 (1.1-3.7) .02</td>
<td>GT + TT 5.1 (1.0-25.4) .03</td>
</tr>
<tr>
<td>rs11787532</td>
<td>8</td>
<td>Zinc finger homeobox 4 (ZFHX4)</td>
<td>Intron 3</td>
<td>C 2.3 (1.0-5.2) .04</td>
<td>CC + CG 2.6 (1.1-6.5) .03</td>
</tr>
<tr>
<td>rs2248202</td>
<td>21</td>
<td>Interferon (alpha, beta, and omega) receptor 2 (IFNAR2)</td>
<td>Intron 1</td>
<td>C 1.9 (1.0-3.7) .04</td>
<td>CC 5.4 (1.1-26.2) .02</td>
</tr>
</tbody>
</table>

Abbreviations: AMPA, alpha-amino-3-hydroxy-5-methyl-4-isoxazole-proprionic acid; CI, confidence interval; OR, odds ratio; SNP, single-nucleotide polymorphism.

*Haldane correction applied to calculation.
little evidence exists in the literature regarding the function of the zinc finger proteins ZFAT and ZFHX4 and the guanosine triphosphatase–activating protein STARD13, and their possible association with response to treatment is unknown to date. Finally, the intergenic SNPs that were validated in the replication cohort may be located in genomic regions that are in high linkage disequilibrium with genes involved in response to interferon beta therapy.

In conclusion, our findings are in line with previous evidence suggesting a role of neurotransmitter-gated channels in response to interferon beta therapy. Furthermore, our study supports a role of genes directly associated with the type 1 interferon pathway and their potential connection with the glutamatergic system in response to interferon beta therapy. However, it is unknown whether these findings are associated with response to interferon beta therapy or reflect the natural history of the disease because of the lack of a placebo group of untreated patients with MS followed up for at least 2 years. Finally, additional replication studies will be needed to further implicate these genes in response to interferon beta therapy in patients with MS.

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Author Affiliations: Centre d’Esclerosi Múltiple de Catalunya, Unitat de Neuroinmunología Clínica, Hospital Universitari Vall d’Hebron, Departament de Medicina de la Universitat Autònoma de Barcelona (Drs Comabella, Río, and Montalban and Ms Fernández), Departament de Ciències Experimentalis i de la Salut, Universitat Pompeu Fabra (Mr Morcillo-Suárez and Dr Navarro), Instituto Nacional de Bioinformática (Mr Morcillo-Suárez), and Institució Catalana de Recerca i Estudis Avançats (Drs Navarro and Martin), Barcelona, Spain; and Neurogenomics Division, Translational Genomics Research Institute, Phoenix, Arizona (Mr Craig). Dr Martin is now with the Institute for Neuroimmunology and Clinical Multiple Sclerosis Research, Center for Molecular Neurobiology Hamburg, University Medical Center Eppendorf, Hamburg, Germany.

Correspondence: Manuel Comabella, MD, Centre d’Esclerosi Múltiple de Catalunya, Unitat de Neuroinmunología Clínica, Hospital Universitari Vall d’Hebron, Edifici Escola d’Infermeria 2ª planta, Passeig Vall d’Hebron 119-129, 08035 Barcelona, Spain (mcomabel@ir.vhebron.net).

Author Contributions: Drs Comabella and Craig had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. Drs Comabella and Craig contributed equally to this work. Study concept and design: Comabella, Craig, and Montalban. Acquisition of data: Craig. Analysis and interpretation of data: Comabella, Craig, Morcillo-Suárez, Río, Navarro, Fernández, and Martin. Drafting of the manuscript: Comabella, Craig, Morcillo-Suárez, Río, Navarro, and Martin. Critical revision of the manuscript for important intellectual content: Comabella, Craig, Río, Navarro, Fernández, Martin, and Montalban. Statistical analysis: Craig, Morcillo-Suárez, Río, Navarro, and Martin. Study supervision: Comabella, Craig, and Montalban.

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