A Pharmacogenetic Study Implicates *SLC9A9* in Multiple Sclerosis Disease Activity

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Objective: A proportion of multiple sclerosis (MS) patients experience disease activity despite treatment. The early identification of the most effective drug is critical to impact long-term outcome and to move toward a personalized approach. The aim of the present study is to identify biomarkers for further clinical development and to yield insights into the pathophysiology of disease activity.

Methods: We performed a genome-wide association study in interferon-β (IFNβ)-treated MS patients followed by validation in 3 independent cohorts. The role of the validated variant was examined in several RNA data sets, and the function of the presumed target gene was explored using an RNA interference approach in primary T cells *in vitro*.

Results: We found an association between rs9828519^{G} and nonresponse to IFNβ ($p_{discovery} = 4.43 \times 10^{-8}$) and confirmed it in a meta-analysis across 3 replication data sets ($p_{replication} = 7.78 \times 10^{-4}$). Only 1 gene is found in the linkage disequilibrium block containing rs9828519: *SLC9A9*. Exploring the function of this gene, we see that *SLC9A9* mRNA expression is diminished in MS subjects who are more likely to have relapses. Moreover, *SLC9A9* knockdown in T cells *in vitro* leads an increase in expression of IFNγ, which is a proinflammatory molecule.

Interpretation: This study identifies and validates the role of rs9828519, an intronic variant in *SLC9A9*, in IFNβ-treated subjects, demonstrating a successful pharmacogenetic screen in MS. Functional characterization suggests that *SLC9A9*, an Na^{+}-H^{+} exchanger found in endosomes, appears to influence the differentiation of T cells to a proinflammatory fate and may have a broader role in MS disease activity, outside of IFNβ treatment.

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It is known that treatment response in multiple sclerosis (MS) patients is highly heterogeneous, encompassing stabilization of disease in some patients and continued disease activity in others despite therapy.[1] Interferon-β

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(IFNβ) is a first-line disease-modifying therapy (DMT) for relapsing–remitting MS (RRMS), with demonstrated beneficial effects in reducing clinical and radiologic disease activity.[2] Nonetheless, in the placebo-controlled clinical trials for IFNβ1a, approximately 65% of RRMS patients assigned to active therapy experienced at least 1 relapse during the first 2 years of treatment.[2–4] Moreover, in clinical practice, we see many patients who have a suboptimal response to DMTs, with the proportion of nonresponders going from 10% to 50%, depending on the definition of treatment response that is applied.[5] As a chronic and progressive disease with an increasing number of treatments, MS is poised for a more personalized approach to intervention that would bring us closer to the goal of suppressing all evidence of disease activity.[6,7]

Here, we used an unbiased assessment of the role of genetic variation found throughout the genome to identify meaningful biomarkers for further clinical development and to yield insights into the pathophysiology of disease activity. We provide robust evidence of replication for the most associated variant, rs9828519, which is intronic to a gene encoding a sodium/hydrogen exchanger (*SLC9A9*) that we implicate as a regulator of proinflammatory lymphocyte activation and MS disease activity.

Subjects and Methods

Italian Collection

The patients involved in the genome-wide association study (GWAS; n = 146) were enrolled at San Raffaele Hospital (OSR; Milan, Italy), and were originally collected for the Wellcome Trust Case Control Consortium 2 (WTCCC2) project.[8] Additional patients were enrolled in the same hospital for the replication phase (n = 275) and to assess the effect of the most associated genetic variant on glatiramer acetate (GA) treatment response (n = 157). All subjects met the McDonald diagnostic criteria.[9] Respectively 12.2% and 13.2% of IFN-treated MS patients in the discovery and replication cohort developed anti-IFNβ neutralizing antibodies (NAb), but no difference was observed between responders and nonresponders ($p = 0.57$ and 0.85, respectively). To be included in the study, patients had to have RRMS, to be treated with IFNβ (Betaferon, Avonex, or Rebif) or with GA as first DMT for at least 6 months, and to have at least 2 years of follow-up. Demographic, clinical, and neuroradiological data were gathered retrospectively, using the iMed database as the source of clinical data. Neuroradiological data were routinely collected at 1 and 2 years of follow-up following drug start. The study was approved by the local ethics committee; written informed consent was obtained from all participants.

American Collection

Clinical and genetic data were available for 557 MS patients enrolled at the Brigham and Women's Hospital (BWH) MS center (Boston, MA). Of them, 281 were treated with IFNβ, whereas 189 were treated with GA and satisfied the same criteria presented in the previous paragraph. Information was collected from the electronic Longitudinal Medical Record at BWH and the Partners Healthcare Multiple Sclerosis clinical research database. Patients who started the treatment before electronic records were available were discarded. Information on NAb was not available in this cohort. The study was approved by the institutional review board of Partners Healthcare, and each patient signed written informed consent for participation in the study.

French Collection

From 2006 to 2008, 325 patient were collected from 7 academic MS centers in France (Caen, Dijon, Nancy, Nice, Paris-La Salpétrière, Rennes, and Toulouse). All Expanded Disability Status Scale scores were confirmed within 3 months of a relapse. Clinical parameters were evaluated according to standardized procedures. No information on NAb was available. Patients were included according to the previous Italian and American cohorts. All subjects gave their informed consent, and the ethics committee of Paris-Pitié-Salpêtrière-France approved the study.

Response Definition

We defined 3 categories of response (responders, nonresponders, and partial responders) based on clinical and magnetic resonance imaging (MRI) data.[10] Specifically, clinical response was assessed as follows: responders, no relapses and no new T2 lesions or Gd-enhancing lesions in 2 years of follow-up; nonresponders, either decrease in the annualized relapse rate of $<50\%$ compared with 2 years before treatment, or ≥ 2 cumulative T2 or Gd-enhancing lesions at 1st-year MRI or ≥ 4 cumulative T2 or Gd-enhancing lesions at 2nd-year MRI or 1st- + 2nd-year MRI; partial responders, patients who did not fulfill the definition of responder or nonresponder.

Genotyping and Quality Control

The Italian IFNβ- and GA-treated MS subjects included in the discovery study were genotyped on the Illumina (San Diego, CA) Human 660-Quad platform, within the WTCCC2 project.[8] The Italian GA-treated patients were typed with the Illumina 2.5M platform. Standard quality control (QC) pipeline for subjects (genotype success rate > 0.99, sex concordance, inbreeding coefficient) and single nucleotide polymorphisms (SNPs; genotype call rate > 0.99, minor allele frequency (MAF) > 0.01, Hardy–Weinberg equilibrium $p > 1 \times 10^{-6}$) was applied. EIGENSOFT was used to remove outliers.[11]

The patients recruited at BWH were genotyped on the Affymetrix (Santa Clara, CA) Human SNP Array 6.0 at the Broad Institute (Cambridge, MA). Details related to the quality parameters used for these data are described elsewhere.[12] The Italian and French samples included in the replication phase were genotyped using the Taqman platform at OSR.

Statistical Analysis

In the GWAS, all SNPs were tested for association with response using PLINK-v1.07.[13] Statistical significance of

individual genotype data was calculated as an allelic chi-square test; odds ratios (ORs) and 95% confidence intervals (CIs) are reported. To evaluate the robustness of the observed distribution of the test statistic, the genomic inflation factor[14] was calculated.

The same statistical analyses were applied also in the replication phase; specifically, PLINK-v1.07[13] was used for measuring MAF and evidence of deviation from Hardy–Weinberg equilibrium within each cohort.

A meta-analysis across the replication and the GA-treated data sets was performed, assuming a per-allelic model. The minor allele was selected as the reference allele and the analyses generated the OR per copy of the reference allele. We synthesized data using a fixed-effect meta-analysis approach.[15]

Cis Expression Quantitative Trait Loci Analysis

Cis expression quantitative trait loci (eQTL) results were obtained from $CD14^+$ monocytes and $CD4^+$ T-cells data of healthy subjects generated by the ImmVar project; details of the subjects, data, and methods utilized are outlined in a recent report.[16] We also report *ex vivo* expression data on peripheral blood mononuclear cells (PBMC) collected from subjects with demyelinating disease who were untreated, treated with GA, or treated with IFNβ ($n = 232$).[12,17] Between July 2002 and October 2007, PBMC samples were collected from RRMS and clinically isolated syndrome (CIS) subjects as part of the Comprehensive Longitudinal Investigation of MS at Brigham and Women's Hospital (CLIMB) in Boston.[18] CIS subjects have only 1 clinical episode of demyelination, whereas MS subjects must have at least 2 of these episodes or 1 clinical event and evidence of disease activity in a paraclinical measure such as MRI.[9] Nonetheless, these two clinical categories share common pathophysiologic events and are also treated in the same manner in the clinical environment. This is why for this analysis we pooled together the RNA data from RRMS and CIS individuals. Some of these subjects were treated with an immunomodulatory drug (GA or IFNβ) at the time of the recruitment. Given the limited number of subjects, we pooled all individuals receiving one of the IFNβ formulations (IFNβ1a intramuscular, IFNβ1a subcutaneous [SC], or IFNβ1b SC) into a single IFNβ category. None of these subjects was on combination therapy for MS at the time of sampling. PBMC isolation, RNA extraction and quantification, and QC steps are described in detail in a prior publication[12]; the expression data based on a U133 v2.0 array are available on the Gene Expression Omnibus (GEO) website. Spearman rank correlation implemented in R (www.r-project.org) was used for the eQTL analysis.

Stimulation of PBMC with IFNβ and Gene Expression Assays

A total of 20 Italian healthy controls (10 $rs9828519^{AA}$ and 10 $rs9828519^{GG}$) were enrolled at OSR to perform whole genome gene expression assays. PBMC were isolated through density gradient centrifugation via Lymphoprep (Axis-Shield Diagnostics, Dundee, UK) following the manufacturer's protocol. The PBMC were incubated at 37°C in RPMI 1640 (Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum (Invitrogen), 1% L-glutamine, and 1% penicillin-streptomycin in 24-well plates (10^6 cells/ml) for 18.5 hours in basal condition or stimulated with 100 IU/ml of IFNβ (Rebif44; Merck Serono, Darmstadt, Germany). Each experiment was performed in triplicate. Total RNA was extracted using TRIzol reagent (Sigma-Aldrich, St Louis, MO) according to manufacturer's protocol. RNA quantification and integrity were assessed using a Nanodrop-2000 spectrophotometer and RNA 6000 Nano Kit on an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA) or gel electrophoresis. If necessary, a DNase (Qiagen, Valencia, CA) digestion was included to remove genomic DNA. The triplicates were pooled to reach the final number of 40 samples (20 basal and 20 IFNβ-stimulated). The mRNA expression profile was obtained using the Illumina Total Prep RNA Amplification Kit (Ambion, Austin, TX) and the HumanHT-12 Expression BeadChip following the manufacturer's instructions. BeadChips were imaged using the Illumina BeadArray Reader, and Illumina GenomeStudio software was used to assess fluorescent hybridization signals. The QC and preprocessing steps (normalization and filtering) were performed using the R statistical environment and the related Bioconductor package Lumi.[19] As QC, a principal component analysis was performed to evaluate outlier samples. The raw data were then quantile normalized. Next, a filter to remove probes close to background was applied, retaining in the analyses all probes that were detected as present by the GenomeStudio algorithm (detection call with $p \leq 0.05$) in >10% of the entire data set. The validation of the array measures of *SLC9A9* gene expression was performed using quantitative real-time polymerase chain reaction (PCR) on ViiA7 (Life Technologies, Carlsbad, CA) with fast SYBR Green chemistry following the manufacturer's protocol (annealing temperature = 57°C) and using the following primer pairs: β-actin, forward primer 5′-CGTCTTCCCCTCCATGT-3′ and reverse primer 5′-GAAGGTGTGGTGCCAGATTT-3′; SLC9A9, forward primer 5′-GCGTGGACCTGGATGAAAA-3′ and reverse primer 5′-TTATTTGCTTCCTGGTGTTGTGA-3′.

For each *in vitro* stimulated triplicate, the relative expression, taking an arbitrary reference sample as 1, was calculated from technical triplicates normalized to β-actin with the $2^{-\Delta\Delta Ct}$ method. The relative expression of *SLC9A9* in the 3 biological replicates was then merged into a unique expression value for each subject in both baseline and stimulated conditions.

In Vivo Evaluation of SLC9A9 Induction Mediated by IFNβ in MS Patients

We performed a GEO database search (http://www.ncbi.nlm.nih.gov/geo) to identify studies with available gene expression data on *SLC9A9* in MS patients at different time points of IFNβ treatment. We found 2 independent studies: references GSE24427[20] and GSE26104.[21] In both of them, the gene expression measures were taken using Affymetrix microarrays. For each experiment, the raw data were normalized with robust multichip average using the R package Affy, and *SLC9A9* expression (defined by 2 probe sets: 227791_at and 242587_at merged) was evaluated comparing the value before and after

IFNβ administration (1 month for GSE24427 and 3 months for GSE26104). For the GSE26104 study, the information about the presence of IFN NAb was available, and only NAb-negative patients were used in the analyses.

Evaluation of the Relation between SLC9A9 and Disease Activity

For these analyses, we used a larger set of expression profiles from PBMC of 363 subjects with RRMS from the CLIMB study in Boston.[17] Subjects in the cis eQTL analysis represent a random subset of these subjects: those who have genome-wide genotype data. Only patients who were untreated or treated with 1 of the 2 first-line disease-modifying treatments, IFNβ and GA, at the time of sampling are in the analysis. Non-negative matrix factorization consensus clustering was used to find the model that best fit the molecular structure in each of the 3 data sets. The algorithm identified 2 clusters of individuals (MS_A and MS_B). For each individual, the time to the first clinical or neurological event was correlated with the classification of the unsupervised clustering using a Cox Proportional Hazards model. Detailed information about this cohort and the methods used to perform the unsupervised classification of the samples is available in Ottoboni et al.[17] Differential expression of SLC9A9 between MS_A and MS_B was calculated for the individuals in each treatment group.

In Vitro T Helper Cell Differentiation and Cytokine Assay

Peripheral venous blood was obtained from healthy volunteers in compliance with institutional review board protocols. PBMC were separated by Ficoll-Paque PLUS (GE Healthcare, Piscataway, NJ) gradient centrifugation. Total $CD4^+$ T cells were isolated from fresh PBMC by negative selection via the $CD4^+$ T-cell isolation kit II (Miltenyi Biotec, Auburn, CA) followed by flow cytometry sorting for $CD45RO^-CD62L^+$ cells. Naive $CD4^+$ cells were cultured in 96-well round-bottom plates (Costar, Cambridge, MA) at 10^4 cells/well in serum-free X-Vivo 15 medium (BioWhittaker, Walkersville, MD), and stimulated with plate-bound anti-CD3 (UCHT1, 1μg/ml) and anti-CD28 (28.2, 1μg/ml) antibodies for 4 to 5 days in the presence of anti-IFNγ and anti–interleukin (IL)$-$4 for T helper (Th)0, IL-12 (10ng/ml), and anti–IL-4 for Th1, and transforming growth factor β (5ng/ml), IL-1β (12.5ng/ml), IL-6, IL-21, and IL-23 (all at 25ng/ml) for Th17 cells. NAb were used at 10μg/ml. Cytokine expression of live $CD4^+$ T cells (7AAD-negative cells) was analyzed by intracellular flow cytometry staining using fluorochrome-conjugated antibodies against IFNγ and IL-17A and confirmed by Luminex bead-based assay (Millipore, Billerica, MA). The Mann–Whitney test was used for flow cytometry cytokine expression analysis between individuals by Prism software v5 (GraphPad Software, San Diego, CA). Data are considered statistically significant at $p < 0.05$. RNA from T cells was purified using a Stratagene RNA kit and transferred directly into the reverse transcriptase reagent using the Applied Biosystems Taqman reverse transcriptase reagents. Samples were subjected to real-time PCR analysis on a PRISM 7000 Sequencer Detection System (Applied Biosystems, Foster City, CA) under standard conditions. Genes analyzed were detected using commercially available assays (Applied Biosystems). Relative mRNA abundance was normalized against β2-microglobulin.

RNA Interference

To knockdown *SLC9A9*, lentivirus particles were produced by cotransfecting pLKO.1 shRNA expression vector with psPAX2 (packaging) and pHEF-VSVG (envelope) plasmids with Lipofectamine 2000 (Life Technologies) into 293T cells (Clontech, Mountain View, CA). Lentivirus supernatant was harvested 48 hours after transfection, filtered through a 0.45μm syringe filter, and centrifuged prior to use. For viral titration, 293T cells were transduced with serial dilutions of the viral supernatants in the presence of Polybrene (5μg/ml) and incubated overnight at 37°C. Twenty-four hours after transduction, viral medium was replaced with regular complete Dulbecco modified Eagle medium and cultures were further incubated at 37°C for 48 hours. Cells were then trypsinized, fixed with paraformaldehyde (4%), and screened for green fluorescent protein (GFP) expression using the FacsCalibur. Viral titer was calculated using the following formula:

$$\text{Titer (transducing units/ml)} = (P\ N)/(D\ V)$$

where P = percentage of GFP^+ cells, N = number of cells per well at the time of transduction, D = dilution factor of sample in the 5 to 20% range, and V = initial volume of viral inoculum.

For SLC9A9 knockdown in $CD4^+$ T cells, freshly isolated naive $CD4^+$ T cells isolated from PBMC of healthy donors by magnetically activated cell sorting negative selection were preactivated with immobilized anti-CD3/CD28 (2μg/ml each), then transduced with optimal titers of lentivirus particles and further incubated for 3 days, followed by drug selection using optimal doses of puromycin for up to 3 days. Antibiotic-resistant $CD4^+$ T cells were used for T helper cell polarization for additional 5 days. Control cells for knockdown were transduced with an shRNA targeting β-galactosidase (LacZ, a nonrelevant gene) used as a negative control. After lentiviral incubation with cells, SLC9A9 protein expression was analyzed by Western blotting using anti-SLC9A9 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) according to standard methods. β-Actin expression, analyzed using anti–β-actin antibody (Sigma, St Louis, MO), was used as internal reference.

Results

Discovery Phase: GWAS

A total of 146 subjects from the San Raffaele collection met the inclusion criteria. These subjects were classified as responders, nonresponders, and partial responders as previously defined.[10] Seventy-four patients met our definition of responders, and 42 were classified as nonresponders. Table 1 summarizes their clinical and demographic characteristics.

After applying stringent QC criteria, we evaluated 539,016 autosomal SNPs for association with IFNβ response. The genomic inflation factor[14] was 0.99,

suggesting that there is no evidence of significant population stratification. Only 1 SNP exceeded the threshold of genome-wide significance ($p < 5 \times 10^{-8}$): the rs9828519^{G} allele is associated with an increased risk of nonresponse to IFNβ therapy ($p = 4.43 \times 10^{-8}$, OR = 5.46, 95% CI = 2.89–10.33; Fig 1A). Cluster plots for this SNP were manually inspected. The proportion of nonresponders increases for each additional copy of the G allele, suggesting an additive effect (see Fig 1B). Rs9828519 is located on chromosome 3q24 and is intronic to *SLC9A9* (see Fig 1C); the regional association plot reveals that there are other SNPs in linkage disequilibrium (LD) with rs9828519 and that they capture the same effect on IFNβ response (rs4839655, $p = 8.14 \times 10^{-5}$; rs12233450, $p = 4.26 \times 10^{-4}$).

Replication Phase

We attempted to replicate the effect of the rs9828519 variant by assessing 3 additional cohorts from the United States, Italy, and France. A total of 281 American MS patients were included in the replication analysis. They were classified as responders by applying the same criteria used in the Italian data set; however, the number of relapses in the 2 years before the treatment start was not available in this data set, so we were not able to distinguish between partial responders and nonresponders. As a consequence, we compared 137 responders to 144 patients who did not match the responder definition. Additional Italian RRMS subjects, recruited independently from the discovery cohort, were used in the replication phase: 125 responders and 150 nonresponders. The third replication stratum consisted of a French cohort composed of 221 responders and 104 nonresponders (see Table 1). No MRI data were available for most of these patients, thus the definition of response applied in the French data set was based on clinical activity. These 3 cohorts are typical of the state of the field in MS, in which there are no very large cohorts that have genetic data paired with detailed treatment outcomes and in which there is a large variability in available data given practical limitations of MRI and clinical data collection. Information on NAb against IFNβ was also not available in the French and US subjects. Overall, these differences in the data available from the different cohorts will tend to reduce statistical power and bias the analysis toward the null. Nonetheless, given the sample size in the meta-analysis, we had >80% power to replicate the association seen for rs9828519^{G} on IFNβ response even if the OR in the replication cohorts is as little as of 1.5.

The results of the association of rs9828519 with treatment response in each stratum were meta-analyzed using a fixed-effect model. Taken together, these 3 data sets confirmed the association of rs9828519 with an increased risk of nonresponse to IFNβ treatment ($p_{replication} = 7.78 \times 10^{-4}$, OR = 1.48, 95% CI = 1.18–1.86, $I^2 = 39\%$; see Fig 1D), with the rs9828519^{G} allele being associated with lower response to IFNβ in all 3 cohorts. Thus, despite the limitations due to varying amount of information available in the different data sets (which bias us toward nonreplication), we observed a consistent direction of effect in all sample collections and provided significant evidence for replication of the effect of rs9828519.

Role in Treatment Response versus Disease Course

Based on the replication results, the rs9828519^{G} allele is associated with the likelihood of increased disease activity while on IFNβ therapy. To evaluate whether the effect may be treatment-specific, we assessed the role of the rs9828519^{G} allele in subjects treated with GA. Two data sets were available for this study (see Table 2): 157 Italian MS cases (91 responders, 66 nonresponders) and 189 American MS patients (98 responders, 91 nonresponders). Given that rs9828519 is not included in the Illumina 2.5M array used to type the Italian GA-treated patients, a SNP in perfect LD with rs9828519, rs2800 ($r^2 = 1$, $D' = 1$), was interrogated. A fixed-effect meta-analysis combined the results and revealed that this genetic variant is not significantly associated with GA treatment response ($p_{combined} = 0.65$, OR = 1.08, 95% CI = 0.77–1.53), although the direction of effect is the same observed also in the IFNβ-treated cohorts. In this GA combined analysis, we had 80% power to replicate the effect size seen for rs9828519^{G} on IFNβ response in the replication meta-analysis (OR = 1.48).

SLC9A9 Expression and IFNβ Stimulation

The rs9828519 variant is intronic to *SLC9A9*, which is the only gene within the LD block that contains the association with disease activity. None of the known variants in strong LD with the leading SNP are coding variants. We therefore explored the possibility that the variant influences gene expression. We first leveraged existing genotype and expression data from purified $CD14^{+}$ monocytes (n = 211) and $CD4^{+}$ T cells (n = 213) extracted from healthy European-American subjects as part of the ImmVar project[16] and a data set generated from PBMC of subjects with demyelinating disease who were untreated, treated with GA, or treated with IFNβ (n = 232).[12,17] Because of the probe design, these subjects have data from only a subset of *SLC9A9* exons, which are used to generate gene-level expression level. In these data, rs9828519 demonstrated no

TABLE 1. Demographic and Clinical Characteristics of the Responder and Nonresponder IFNβ-Treated Patients Included in the Study

	Discovery		Replication					
	Italy		USA		Italy		France	
Characteristics	R, n = 74	NR, n = 42	R, n = 137	NR, n = 144	R, n = 125	NR, n = 150	R, n = 221	NR, n = 104
F:M	52:22	34:8	102:35	108:36	81:44	108:42	171:50	79:25
Age at disease onset, yr (range)	28.5 (17.2–52.6)	28.2 (14.5–55.1)	31.7 (14.9–53.7)	31.5 (12.0–53.9)	30.4 (13.8–60.0)	27.6 (10.9–54.0)	29.7 (13.0–52.0)	27.3 (12.0–51.0)
Age at treatment start, yr (range)	34.4 (18.8–56.8)	35.6 (20.2–57.8)	36.7 (17.3–63.6)	36.1 (16.0–63.2)	35.1 (16.9–63.8)	33.1 (15.1–60.7)	34.3 (15.0–58.0)	33.1 (15.0–55.0)
EDSS at treatment start (range)	1.9 (0.0–4.0)	2.1 (0.0–5.0)	1.55 (0.0–4.0)	1.7 (0.0–4.5)	1.7 (0.0–4.0)	1.9 (0.0–4.0)	1.9 (0.0–6.0)	2.2 (0.0–6.0)
Disease duration, yr (range)	5.8 (0.3–31.1)	7.4 (0.6–22.5)	5.0 (0.3–35.4)	4.6 (0.3–42.4)	4.7 (0.3–21.1)	5.5 (0.2–28.0)	4.6 (0.3–26.0)	5.8 (0.2–25.0)
Type of IFNβ treatment, No. [%]								
1a IM Avonex	25 [33.8]	16 [38.1]	103 [75.2]	104 [72.2]	39 [31.2]	53 [35.3]	116 [52.5]	60 [57.7]
1a SC Rebif	36 [48.6]	21 [50.0]	13 [9.5]	27 [18.8]	79 [63.2]	87 [58.0]	57 [25.8]	16 [15.4]
1b SC Betaferon	13 [17.6]	5 [11.9]	21 [15.3]	13 [9.0]	7 [5.6]	10 [6.4]	48 [21.7]	28 [26.9]

EDSS = Expanded Disability Status Scale; F = female; IFNβ = interferon β; IM = intramuscular; M = male; NR = nonresponders; R = responders; SC = subcutaneous.

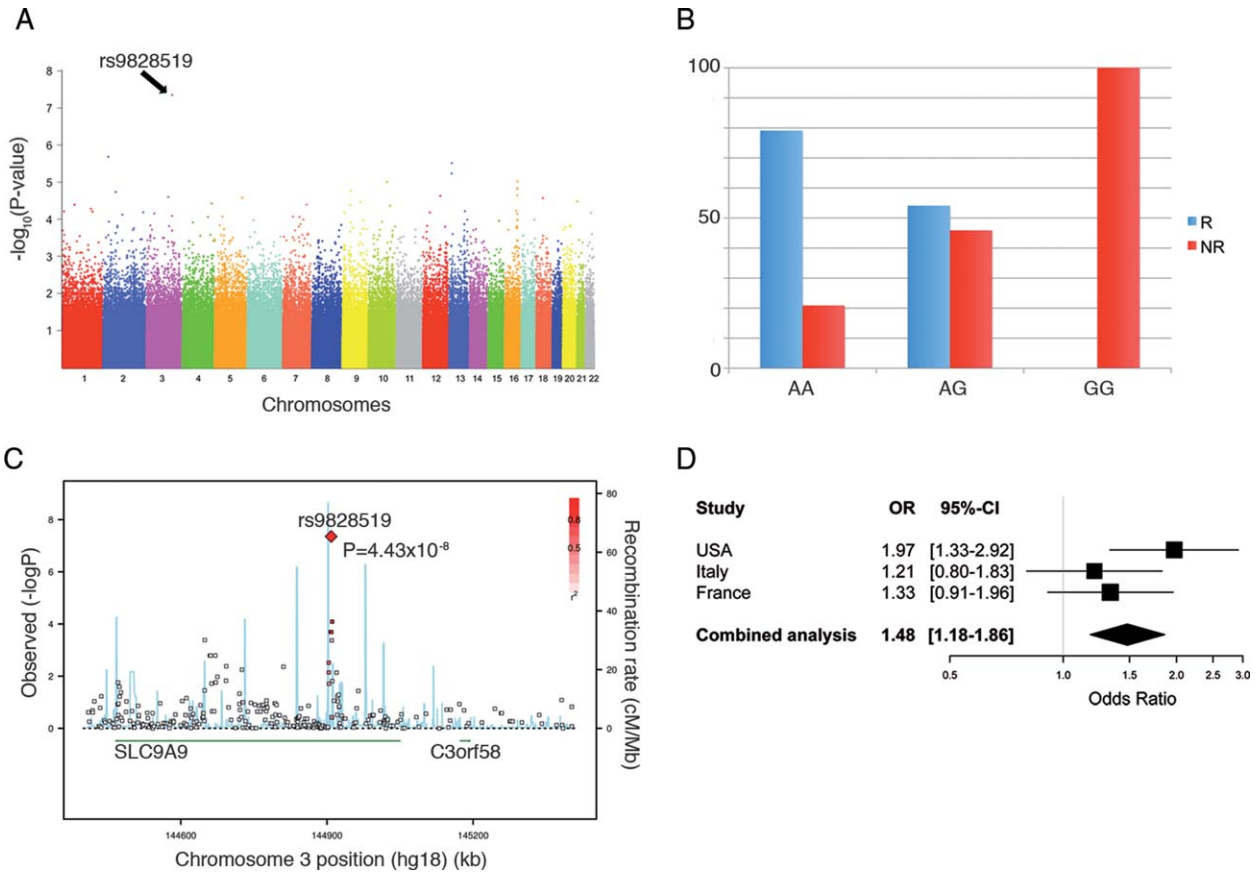

FIGURE 1: Rs9828519 is associated with nonresponse to interferon-β (IFNβ) therapy. (A) Overview of the results in the discovery Italian collection. Probability values (shown as −log10 values) from the discovery genome-wide association study are plotted across the genome. The rs9828519 variant on chromosome 3 is the only one that reached genome-wide significance. The frequency of the minor allele G is 0.38. The G allele is associated with nonresponse to IFNβ therapy. (B) Proportion of responders (R) and nonresponders (NR) across the 3 rs9828519 genotypes in the Italian IFNβ-treated patients. The rs9828519^{G} allele is associated with increased risk of nonresponse to IFNβ therapy ($p = 4.43 \times 10^{-8}$, odds ratio [OR] = 5.46, 95% confidence interval [CI] = 2.89–10.33). All subjects who are carriers of the GG genotype are nonresponders. An additive effect is observed; the proportion of nonresponder patients increases for each additional copy of the G allele that is carried, going from 20.9% in AA individuals, to 45.9% in AG individuals, to 100% in GG individuals. (C) Regional association plot for the *SLC9A9* locus. The *SLC9A9* locus is illustrated, with the local recombination rate plotted over a 1,000kb chromosomal segment centered on rs9828519. Each symbol represents 1 single nucleotide polymorphism (SNP) found in this locus; the most associated SNP in the responder versus nonresponder analysis, rs9828519, is marked by a red symbol. The color of each symbol is defined by the extent of linkage disequilibrium with rs9828519: red ($r^2 > 0.8$), orange ($0.8 > r^2 > 0.5$), gray ($0.5 > r^2 > 0.3$) and white ($r^2 < 0.3$). Physical positions are based on build 36 of the human genome. (D) Meta-analysis across the replication data sets. The forest plot summarizes the results obtained for rs9828519 in the American, Italian, and French replication cohorts. Summary ORs and respective 95% CIs are calculated using the fixed-effect method.

association with *SLC9A9* expression (data not shown). Although these data sets have a moderate sample size and reasonable power, they sample only the *ex vivo* context of the sampled cell populations, and these results do not exclude the possibility that rs9828519 has an effect in a specific cell state or in a specific cellular subpopulation that has not been sampled.

To address this issue, in part, we assessed whether the expression of *SLC9A9* is regulated by IFNβ by stimulating *in vitro* PBMC collected from healthy controls. A significant increase in *SLC9A9* expression was observed after IFNβ stimulation (paired *t* test *p* value $= 4.01 \times 10^{-7}$; mean induction [stimulated/baseline] = 1.26; Fig 2A), but there was no difference in terms of induction between rs9828519^{AA} and rs9828519^{GG} subjects ($p = 0.40$; mean induction in rs9828519^{AA} vs rs9828519^{GG} = 1.29 vs 1.23; see Fig 2B). These results were validated by quantitative real-time PCR ($p = 2.1 \times 10^{-3}$, mean induction = 1.27). The induction of *SLC9A9* after IFNβ stimulation was also confirmed *in vivo*, using gene expression data from 2 publicly available independent experiments (references: GSE24427[20] and GSE26104[21]). Specifically, when assessing the *SLC9A9* expression profile in MS patients before the first IFNβ administration and after treatment, an upregulation of *SLC9A9* was observed ($p = 3.8 \times 10^{-3}$ and 0.030 respectively; see Fig 2C, D).

TABLE 2. Demographic and Clinical Characteristics of the Italian and American Glatiramer Acetate–Treated Patients Included in the Study

Characteristics	Italy		USA	
	R, n = 91	NR, n = 66	R, n = 98	NR, n = 91
F:M	58:33	51:15	78:20	72:19
Age at disease onset, yr (range)	31.9 (13.2–54.5)	27.8 (13.6–45.3)	34.6 (13.5–58.3)	34.0 (17.9–48.6)
Age at treatment start, yr (range)	36.3 (13.4–62.2)	35.7 (13.7–56.7)	40.8 (19.5–66.8)	38.6 (18.2–60.4)
Disease duration, yr (range)	7.5 (0.2–32.9)	5.6 (0.1–22.1)	6.2 (0.3–30.0)	4.7 (0.3–37.2)

F = female; M = male; NR = nonresponders; R = responders.

Relation of SLC9A9 to Disease Activity and T-Cell Differentiation

To further explore the role of *SLC9A9* in MS, we examined its level of expression in 2 subsets of subjects with demyelinating disease,[17] 1 of which (the MS_A subset) is more likely to have a relapse or new evidence of disease activity on MRI when compared to the MS_B subset. The subsets are defined using an RNA signature from PBMC that does not contain *SLC9A9* and are present in each of the 3 groups of patients that were sampled: subjects treated with GA, subjects treated with IFNβ, and untreated subjects. We see that *SLC9A9* RNA expression is reduced in subjects classified as MS_A in all 3 groups ($p < 10^{-5}$; Fig 3). Thus, *SLC9A9* appears to be downregulated in PBMC samples from MS subjects who have a more activated lymphocyte profile and are at an increased risk of another demyelinating event.[17]

To examine this question in greater detail, we evaluated *SLC9A9* expression in data collected as part of the ImmVar project.[16] Here, we delineated transcriptome-wide changes in gene expression in naive $CD4^+$ T cells as they differentiate into Th17 (IL-17^+) cells, a subset of proinflammatory T cells that plays an important role in MS inflammatory events. Consistent with the PBMC results, we see that *SLC9A9* expression is reduced during the differentiation process as naive $CD4^+$ T cells become polarized into Th17 cells (data not shown). To confirm these results, we repeated the experiments and expanded our analysis to include Th1 (IFNγ^+) cells, a proinflammatory T-cell population that is also implicated in MS. We found that *SLC9A9* mRNA expression is decreased as early as 8 hours following the onset of differentiation in both Th1 and Th17 cells polarized *in vitro* according to established protocols[22] (Fig 4A). Interestingly, the rapid decrease in *SLC9A9* expression in Th1 and Th17 cells is maintained up to 48 hours following polarization, suggesting that *SLC9A9* may provide an inhibitory signal of T-cell activation.

Validating the Putative Role of SLC9A9 as a Regulator of T-Cell Function

We analyzed the role of *SLC9A9* in $CD4^+$ T-cell activation and differentiation into Th1 and Th17 cells using an *in vitro* model. Specifically, we asked whether *SLC9A9* gene silencing influences T helper cell development *in vitro*. To address this, we transduced naive $CD4^+$ T cells with lentiviral particles encoding shRNA specific for human SLC9A9 or control vector (LacZ), and cells resistant to puromycin selection were polarized under Th0, Th1, or Th17 cell conditions for 5 days. At the end of this differentiation period, intracellular cytokine expression was measured using flow cytometry. We found that the frequency of $CD4^+$IFNγ^+ but not of $CD4^+$IL-17^+ T cells was more highly induced in all T-cell differentiations compared to LacZ-transduced control cells, suggesting that SLC9A9 is a negative regulator of IFNγ signaling in T cells (see Fig 4B). The efficiency of the SLC9A9 knockdown was confirmed at the protein level by immunoblots of SLC9A9 and β-actin that is shown as an internal control (see Fig 4C). Quantification of the frequency of $CD4^+$IFNγ^+ determined by flow cytometry staining in shRNA-SLC9A9–transduced and control Th1 cells obtained from healthy subjects ($p = 0.008$, n = 7) demonstrates that SLC9A9 knockdown significantly upregulates IFNγ production in polarized Th1 cells (see Fig 4D). SLC9A9 does not influence T-cell survival, as demonstrated in Th1 cells that were transduced with SLC9A9-specific or control shRNA using 7AAD viability staining ($p = 0.16$, n = 7).

Discussion

Until now, targeted and the 2 genome-wide pharmacogenetic studies in MS have reported either a lack of association or weak associations with treatment response, which have not been widely validated.[23–25] Moreover, previous studies suggested that there may not be a common genetic variant with a strong effect on treatment

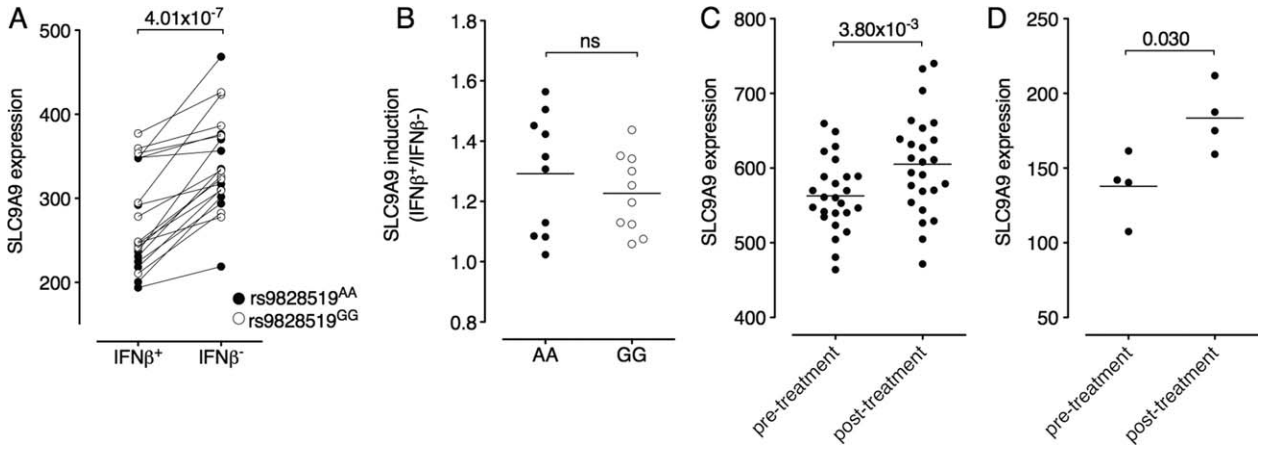

FIGURE 2: ***SLC9A9*** **is upregulated after interferon-*β* (IFN*β*) stimulation. (A, B) The expression of *SLC9A9* gene was measured in peripheral blood mononuclear cells (PBMC) collected from 10 rs9828519^{AA} *(black dots)* and 10 rs9828519^{GG} *(white dots)* healthy controls and stimulated with IFN*β*. (A) An increase in the expression was observed ($p = 4.01 \times 10^{-7}$; mean induction = 1.26) comparing the baseline (IFNβ^-) and the IFN*β*-stimulated (IFNβ^+) conditions for all the samples. (B) No difference in induction, defined as the ratio between the expression after stimulation with IFN*β* and the expression at baseline condition, was observed comparing rs9828519^{AA} and rs9828519^{GG} samples ($p = 0.40$). (C, D) The plot shows the *SLC9A9* expression values in PBMC collected from multiple sclerosis patients before and during the treatment with IFN*β* in 2 independent studies: GSE24427[20] (C) and GSE26104[21] (D). Each dot represents 1 sample (n = 25 in GSE24427; n = 4 in GSE26104). Black horizontal bars represent the mean for each group. ns = not significant.**

response, and our analyses are largely consistent with this putative genetic architecture for IFN*β* response. The main limitations of previous studies were an underpowered sample size and a lack of consistency of response criteria across studies, which prevent direct comparison.[5]

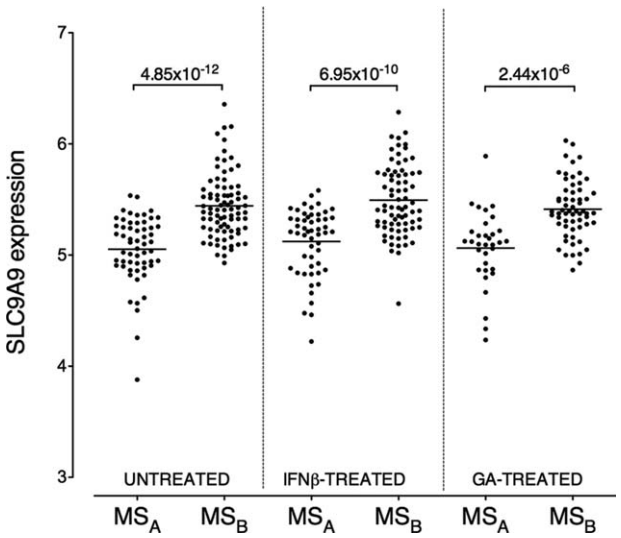

FIGURE 3: ***SLC9A9*** **is downregulated in multiple sclerosis (MS) patients with a more active disease course. The expression of *SLC9A9* gene was measured in 141 MS untreated patients, 128 interferon-*β* (IFN*β*)-treated patients, and 94 glatiramer acetate (GA)-treated patients using the GeneChip human genome U133 Plus.2.0 arrays (Affymetrix, Santa Clara, CA).[17] Each cohort was divided into the MS_A and MS_B groups. Each dot represents an individual. The expression of *SLC9A9* was significantly higher in MS_B compared to MS_A. The data reported are relative to the average of the probes mapping to *SLC9A9* (227791_at and 242587_at) after log2 transformation. The horizontal lines represent the mean.**

Such differences in the definition of response—as in our study—will tend to reduce statistical power and bias replication efforts toward a null result. Here, we report the discovery of a variant that is associated with MS disease activity while on IFN*β* therapy in a small genome-wide study and its replication in a much larger set of subjects gathered from 3 distinct sample collections. The discovery cohort was a subset of individuals initially collected to identify MS susceptibility loci; however, the enrolled patients are representative of overall MS subjects on IFN*β* treatment followed at OSR in terms of baseline demographic and clinical characteristics (data not shown).

Only a single variant was tested at the replication stage, because it was the only variant meeting a genome-wide threshold of significance ($p < 5 \times 10^{-8}$) in the discovery study. As expected with winner's curse, the effect size of the variant is much larger in the discovery study than in the replication study (see Fig 1D). Moreover, the definition of response was not homogenous across the studied cohorts and it could have an impact on the final results. However, the direction of effect is consistent in all 4 data sets, and this observation is unlikely to occur by chance. An overall meta-analysis of all 4 data sets was performed in which a less extreme probability value ($p = 8.39 \times 10^{-7}$) is seen, as expected given the small sample size of the discovery study. This secondary analysis does not detract from the replication of our primary analysis, and we look forward to further replication efforts to strengthen the evidence supporting a role for rs982851. A more definitive assessment of whether this variant affects only IFN*β* response or has a broader role

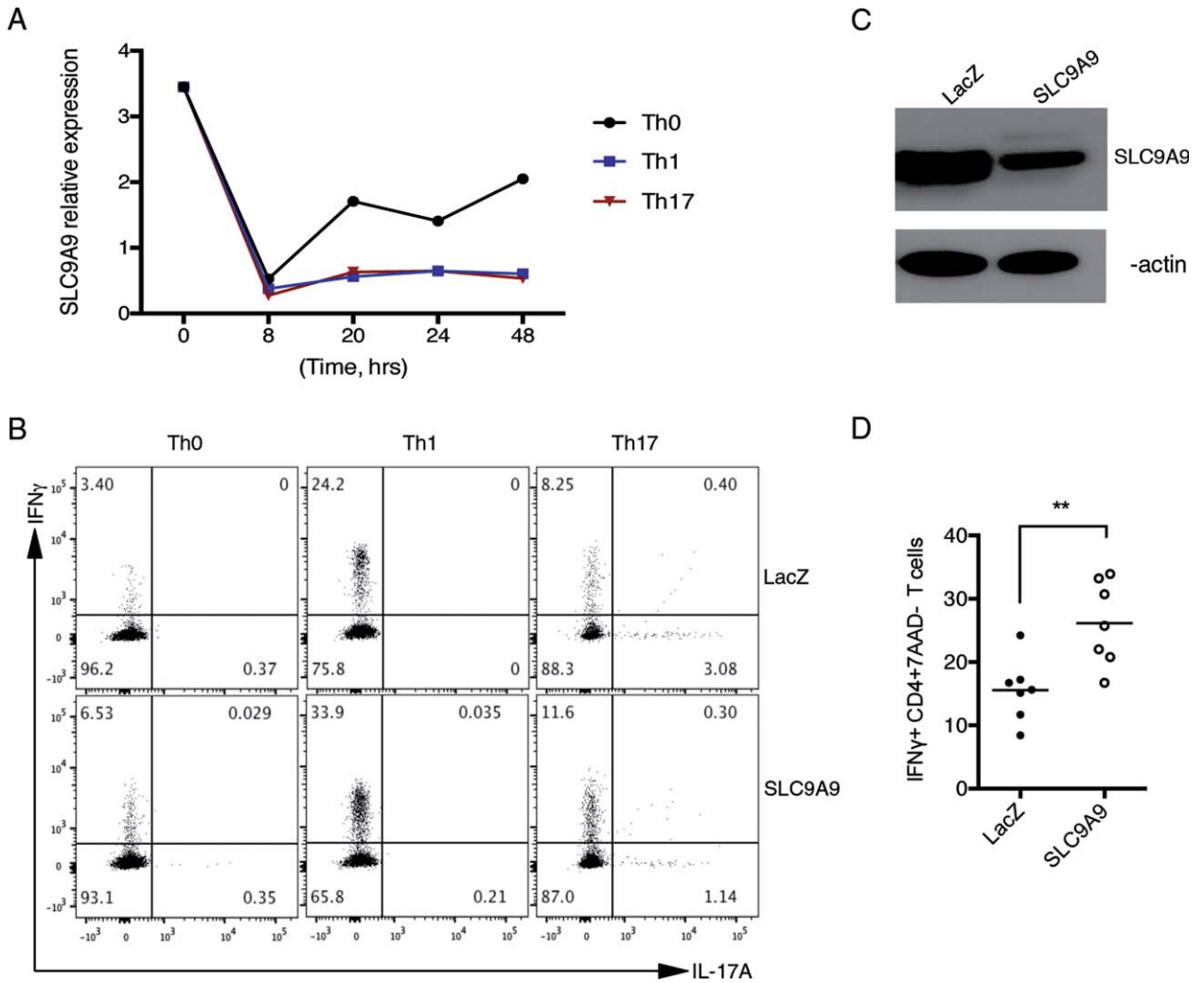

FIGURE 4: SLC9A9 is a negative regulator of CD4$^+$IFNγ^+ T cells. (A) *SLC9A9* expression is rapidly downregulated in polarized T helper (Th) cells. Naive CD4$^+$ T cells were differentiated under Th0, Th1, or Th17 cell conditions for up to 48 hours, and *SLC9A9* mRNA expression was analyzed at each time point by Taqman quantitative polymerase chain reaction. (B) A representative experiment showing the effects of *SLC9A9* gene silencing on interferon-γ (IFNγ) expression in polarized CD4$^+$ T cells. CD4$^+$ T cells were transduced with shRNA specific for SLC9A9 or negative control gene (LacZ) followed by polarization under Th0, Th1, and Th17 conditions. IFNγ and interleukin (IL)−17A expression was assessed by intracellular cytokine staining. (C) Western blot of SLC9A9 and β-actin protein expression in control LacZ and shRNA-SLC9A9 transduced cells. (D) Comparison of the frequency of CD4$^+$IFNγ^+ T cells determined by flow cytometry in control and shRNA-SLC9A9–transduced Th1 cells from healthy donors (n = 7). The horizontal lines represent the mean. **$p < 0.01$ by Student *t* test.

in disease activity awaits additional studies with subjects treated with GA or other MS therapies. Our current analysis suggests that rs9828519 does not have a strong effect on GA treatment response, but the direction of the association signal is the same as that seen with IFNβ, and the expression data from MS_A and MS_B subjects (see Fig 3) suggest that the role of *SLC9A9* may not be limited to IFNβ treatment response. It may be more broadly relevant to disease activity in all MS subjects.

On an individual basis, rs9828519 and other variants that we may find in the future have a modest effect on the probability of treatment response. Are they worth finding? The answer is yes; first, they may point to mechanisms that could be manipulated to achieve larger effects on treatment response. Second, recognizing the effect of this and other variants will play an important role in supporting the identification of other types of biomarkers whose effects will be clearer when confounders such as genetic variants are accounted for. Third, rs9828519 could make a contribution to an estimate of the likelihood of treatment response derived by aggregating the effects of many genetic variants and other fluid or imaging biomarkers. This approach, although not ready for clinical deployment, has been shown to work in differentiating MS subjects from healthy subjects.[26,27] Thus, although it is likely that genetic information will be incorporated into some aspects of disease management in the coming decade, algorithms will clearly require more than

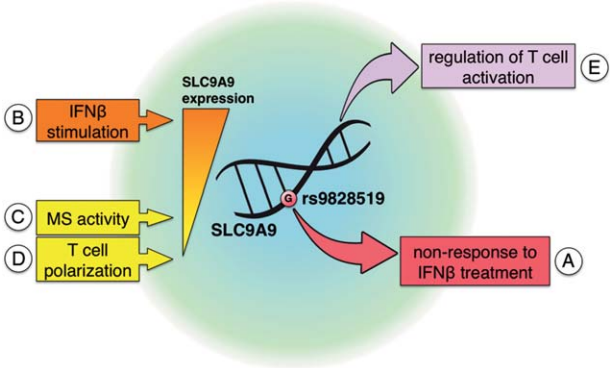

FIGURE 5: Summary of the involvement of rs9828519 and SLC9A9 in response to interferon-*β* (IFN*β*) treatment and T-cell activation. We observed an association of the rs9828519^{G} allele with the nonresponse to IFN*β* treatment in an Italian cohort of IFN*β*-treated multiple sclerosis (MS) patients and further replicate it in 3 additional cohorts (A). Rs9828519 is intronic to *SLC9A9*, a sodium/hydrogen exchanger localized in the sorting/recycling endosomes. We detected a higher expression of *SLC9A9* in peripheral blood mononuclear cells stimulated with IFN*β* both *in vitro* and *in vivo* (B), whereas a lower expression was observed in more activated MS patients (C) and during T-cell polarization (D). Moreover, *SLC9A9* appears to influence the differentiation of T cells (E).

just genetic data, because the dynamic state of the subject is not captured by a static measure of genetic variation.

The rs9828519 SNP is intronic to *SLC9A9*, the only gene found within the LD block that contains rs9828519 making it the most likely candidate causal gene. *SLC9A9* encodes a sodium/hydrogen exchanger that is widely expressed, including in the central nervous system and immune cells (http://www.ebi.ac.uk/gxa/).[28,29] It is localized in the endosomal compartment and regulates intraluminal pH. No information is available in the literature regarding the involvement of *SLC9A9* in MS or in the IFN*β* pathway. Of note, one of the existing IFN*β* pharmacogenetic study had an enrichment of variants in genes related to ion channel pathways,[23] supporting the hypothesis that ion exchange mechanisms may be implicated in treatment response.

To date, we cannot differentiate which of the 10 variants in LD ($r^2 > 0.8$) with rs9828519 is the causal variant. None of them has an obvious mechanistic impact on the *SLC9A9* coding sequence, regulatory elements, or splicing machinery, and we have not yet found evidence that the variant driving the association with disease activity influences levels of RNA expression in *ex vivo* T cells and monocytes of healthy subjects or PBMC of MS patients. The results obtained from *in vitro* experiments on PBMC of MS patients do not show a differential induction of *SLC9A9* in rs9828519^{AA} compared to rs9828519^{GG} individuals. However, these data represent only a very small fraction of the possible immune cell subtypes and cell states in which the variant could express its role, and we cannot exclude that the genetic variant has an effect in a specific cell type or on different RNA isoforms. Therefore, although the variants' mechanism of action remains elusive, we have nonetheless characterized the role of *SLC9A9* in MS and in the Th1 and Th17 cells that play a key role in MS.

The data accumulated to date (Fig 5) implicate SLC9A9 as a molecule that influences T-cell differentiation and activation of multiple T-cell subtypes. Because SLC9A9 affects pH in the endosomal and Golgi compartments of cells,[30] we speculate that such changes could lead to altered glycosylation,[31] an important mechanism in regulating inflammation and MS.[32] This would be consistent with a recent report of *SLC9A9* variants associated with *N*-glycans plasma level.[33] Further studies are now needed to understand the molecular consequences of decreased *SLC9A9* RNA expression that lead to an exaggerated expression of IFNγ. Such studies will also evaluate the role of *SLC9A9* in other immune cell types, given its broad expression pattern.

Overall, starting with a pharmacogenetic approach, we identified and validated the role of rs9828519, an intronic variant in *SLC9A9*, in IFN*β*-treated MS patients. We do not have definitive evidence that this *SLC9A9* variant has a treatment-specific effect; the GA sample size is too small to offer a definitive assessment. The transcriptional studies suggest that *SLC9A9* may be more broadly implicated in disease activity, independent of treatment. Further studies are needed to resolve this issue. The *in vitro* studies that we have performed to understand the function of SLC9A9 yield data suggesting that it is a very interesting candidate regulator of T-cell activation that appears to have a role in MS. We speculate that *SLC9A9* may yield insights for novel therapeutic approaches for the inflammatory component of MS, one (for example, alteration of an Na^+/H^+ exchanger) that have not been targeted to date. Finally, given its role in disease activity in MS and in influencing T-cell activation, it presents an intriguing new molecule for further investigation into other inflammatory diseases such as Crohn disease, rheumatoid arthritis, type 1 diabetes, and other diseases in which T-cell activation plays an important role.

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Authorship

F.E., M.S., F.M.B., and P.L.D.J. designed the study, interpreted the data, and wrote the manuscript. F.E., G.L., C.G., M.Rom., J.M.S., V.M., M.Rod., H.L.W., D.B., G.C., F.M.B., and P.L.D.J. selected subjects for inclusion into the study and characterized the participants. F.E., M.S., L.O., J.M.R., T.R., E.T.L., and W.E. analyzed the data. M.S., J.M.R., P.B., T.P., and W.E. generated the data. G.C., N.A.P., and C.B. helped with data interpretation. T.P. generated the data. All authors have read and reviewed the submitted version of the manuscript. F.E. and M.S. contributed equally to this work. F.M.B. and P.L.D.J. contributed equally to this work.

Potential Conflicts of Interest

F.E.: honoraria, Serono Symposia International Foundation. J.M.S.: personal fees, Biogen Idec, Teva Neuroscience, Genzyme, Novartis. V.M.: personal fees, Bayer Schering Pharma, Biogen-Dompè, Merck Serono, Novartis, Sanofi-Aventis, Teva Pharmaceutical. H.L.W.: grants, EMD Serono, GlaxoSmithKline; consultancy, Biogen Idec, EMD Serono, Genzyme, Teva Pharmaceuticals, Novartis, Nasvax, GlaxoSmithKline. D.B.: personal fees, travel expenses, Bayer Schering Pharma, Sanofi-Aventis, Biogen Idec, Merck Serono, Teva Pharmaceutical. N.A.P.: consultancy, advisory boards, Merck. G.C.: consultancy, advisory boards, speaking fees, Novartis, Teva Pharmaceutical, Sanofi-Aventis, Genzyme, Merck Serono, Biogen, Bayer Schering Pharma, Serono Symposia International Foundation, Excemed, Almirall, Chugai, Receptors. F.M.B.: grants, consultancy, travel expenses, Teva Neuroscience, Biogen Idec, Merck Serono. P.L.D.J.: grants, consultancy, speaking fees, Teva Neuroscience, Biogen Idec, Merck Serono, Vertex.

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