Analysis of Plasminogen Genetic Variants in Multiple Sclerosis Patients

A Dessa Sadovnick, PhD*,†, Anthony L Traboulsee, MD†, Cecily Q Bernales, BSc*, Jay P Ross, BSc*, Amanda L Forwell, BSc*, Irene M Yee, MSc*, Lena Guillot-Noel, MSc†, Bertrand Fontaine, MD, PhD†, Isabelle Cournu-Rebeix, PhD‡, Antonio Alcina, PhD§, Maria Fedetz, PhD§, Guillermo Izquierdo, MD, PhD**, Fuencisla Matesanz, PhD§, Kelly Hilven, MSc††, Bénédicte Dubois, MD, PhD††, An Goris, PhD††, Ianire Astobiza, PhD§§,***, Iraide Alloza, PhD§§,***,++++, Alfredo Antíguedad, MD†††, Koen Vandenbroeck, PhD§§,***,+++ Denis A Akkad§§§, Orhan Aktas****, Paul Blaschke††††, Mathias Buttmann††††, Andrew Chan§§§§, Joerg T Epplen§§§§, Lisa-Ann Gerdes******, Antje Kroner+++++++††††, Christian Kubisch+++++++††††, Tania Kümpfel******††††, Peter Lohse$$$$$$$$, Peter Rieckmann+++++++††††, Uwe K Zettl††††, Frauke Zipp+++++++††††, Lars Bertram$$$$$$$$, Cristina M Lill$$$$$$, Oscar Fernandez, MD+++++++††††, Patricia Urbaneja, MD+++++++††††, Laura Leyva, PhD+++++++††††, Jose Carlos Alvarez-Cermeño, MD$$$$$$, Rafael Arroyo, MD, PhD$$$$$$, Aroa M Garagorri, MSc+++++++††††, Angel García-Martínez MSc+++++++††††, Luisa M Villar, PhD$$$$$$, Elena Urcelay, PhD+++++++††††, Sunny Malhotra, MSc+++++++††††, Xavier Montalban, MD+++++++††††, Manuel Comabella, MD+++++++††††, Thomas Berger, MD$$$$$$, Franz Fazekas, MD$$$$$$††††, Markus Reindl, MD$$$$$$, Mascha C Schmied, MD+++++++††††, Alexander Zimprich, MD+++++++††††, Carles Vilarinío-Güell, PhD††* Medical Genetics, University of British Columbia, Vancouver, British Columbia, V6T 1Z3, Canada† Division of Neurology, Faculty of Medicine, University of British Columbia, Vancouver, British Columbia, V6T 1Z3, Canada‡ Inserm U 1127, CNRS UMR 7225, Sorbonne Universités, UPMC Univ Paris 06 UMR S 1127, Institut du Cerveau et de la Moelle épinière, ICM, Paris, France§ Cell Biology and Immunology, Instituto de Parasitología y Biomedicina López Neyra (IPBLN), CSIC, 18100 Granada, Spain** Unidad de Esclerosis Múltiple, Hospital Universitario Virgen Macarena, 41071 Sevilla, Spain†† Laboratory for Neuroimmunology, Neurosciences, KU Leuven – University of Leuven, Herestraat 49 bus 1022, Leuven, Belgium††† Neurology, University Hospitals Leuven, KU Leuven – University of Leuven, Herestraat 49, Leuven, Belgium§§ Neurogenomiks Group, Universidad del País Vasco (UPV/EHU), 48940 Spain*** Achucarro Basque Center for Neuroscience, 48170 Spain++++ IKERBASQUE, Basque Foundation for Science, 48011 Spain++++ Servicio de Neurología, Hospital Universitario Basurto-Osakidetza, 48940 Bilbao, Spain$$$$ Human Genetics, Ruhr University, 44801 Bochum, Germany***** Neurology, Medical Faculty, Heinrich Heine University, 40225 Düsseldorf, Germany+++++ Neurology, University of Rostock, 18059 Rostock, Germany++++ Neurology, University of Würzburg, 97080 Würzburg, Germany$$$$$ Neurology, St. Josef-Hospital, Ruhr-University, 44791 Bochum, Germany***** Institute for Clinical Neuroimmunology, Ludwig Maximilian University, 80539 Munich, Germany+++++++ Centre for Research in Neuroscience at McGill University, Montreal, H3H 2R9 Canada
Running Title
Plasminogen in multiple sclerosis

Keywords
Multiple sclerosis, Genetics, Linkage; Association; Plasminogen.

Corresponding author: Carles Vilariño-Güell
Address: 2215 Wesbrook Mall, University of British Columbia, Vancouver, BC, V6T 1Z3, Canada
Email: carles@can.ubc.ca
Phone: +1-604-827-1303

Word count: 2947 Abstract: 254 Title characters: 62
References: 31
Tables: 2 Figures: 2
Supplemental Material: 5 Tables and 2 Figures
Multiple sclerosis (MS) is a prevalent neurological disease of complex etiology. Here we describe the characterization of a multi-incident MS family which nominated a rare missense variant (p.G420D) in plasminogen (PLG) as a putative genetic risk factor for MS. Genotyping of PLG p.G420D (rs139071351) in 2160 MS patients and 886 controls from Canada identified ten additional probands, two sporadic patients and one control with the variant. Segregation in families harboring the rs139071351 variant, identified p.G420D in 26 out of 30 family members diagnosed with MS, 14 unaffected parents and 12 out of 30 family members not diagnosed with disease. Despite considerably reduced penetrance, linkage analysis supports co-segregation of PLG p.G420D and disease. Genotyping of PLG p.G420D in 14446 patients and 8797 controls from Canada, France, Spain, Germany, Belgium and Austria failed to identify significant association with disease (p=0.117), despite an overall higher prevalence in patients (OR=1.32; 95% CI=0.93-1.87). To assess whether additional rare variants have an effect on MS risk, we sequenced PLG in 293 probands and genotyped all rare variants in cases and controls. This analysis identified nine rare missense variants, and although three of them were exclusively observed in MS patients segregation does not support pathogenicity. PLG is a plausible biological candidate for MS owing to its involvement in immune system response, blood-brain barrier permeability and myelin degradation. Moreover, components of its activation cascade have been shown to present increased activity or expression in MS patients compared to controls; further studies are needed to clarify whether PLG is involved in MS susceptibility.
INTRODUCTION

Multiple sclerosis (MS) is a chronic inflammatory demyelinating and neurodegenerative disease of the central nervous system. The genetic contribution to disease susceptibility has been demonstrated in family and twin studies (Ebers et al. 1986; Sadovnick 1993; Fagnani et al. 2015), and the first pathogenic mutation for MS has been recently identified in NR1H3 (Wang et al. 2016). In addition, a large number genetic risk factors, primarily related to the immune system, have already been identified through association studies (Beecham et al. 2013; Sawcer et al. 2011). However, with the exception of HLA-DRB1, all associated variants have a minor effect on overall disease. The identification of genetic components of major effect on disease development is paramount for the generation of physiologically relevant cellular and animal models of human disease, and the generation of treatment strategies that address the underlying biological mechanisms responsible for the onset of MS.

MATERIAL AND METHODS

Participants

A total of 2160 MS patients and 886 unrelated healthy controls from Canada, which includes 1857 multi-incident families, collected through the Canadian Collaborative Project on the Genetic Susceptibility to Multiple Sclerosis (CCPGSMS) were included in this study.(Sadovnick et al. 1998). Five independent European cohorts consisting of 2391 MS patients and 672 healthy controls from France, 4288 patients and 4018 controls from Spain, 3733 patients and 2722 controls from Germany, 1006 patients and 504 controls from Belgium and 925 patients from Austria were used for replication. All patients were diagnosed with MS according to published criteria (Poser et al. 1983; McDonald et al. 2001; Polman et al. 2005), and the demographics for each cohort are presented in Table 1. The ethical review board at each institution approved the study, and all participants provided written informed consent.

Exome sequencing

We performed exome sequencing in three patients diagnosed with MS (pedigree A; II-1, II-4 and III-1) from a multi-incident family (Figure 1). Exonic regions were enriched using an Ion AmpliSeq exome kit (57.7Mb) and sequenced in an Ion Proton sequencer (Life Technologies, Carlsbad, CA, USA) with a minimum average coverage of 50 reads per base and an average read length of 150 bases. The Ion Torrent Server v4 was used to map reads to NCBI Build 37.1 reference genome using the Torrent Mapping Alignment Program (TMAP) and to identify variants differing from the reference. Sequences with a mapping Phred quality score under 20,
fewer than five reads or over 95% strand bias were excluded from further analysis.

**Sequencing, genotyping and statistical analysis**

Sanger sequencing was used to genotype amplicons containing exome variants of interest and all 19 coding exons and exon-intron boundaries of plasminogen (*PLG, NM_000301.3*) by polymerase chain reaction (PCR) as previously described (Sadovnick et al. 2013). Nine tagging SNPs (tSNPs) spanning a 61 kb region encompassing the *PLG* locus were selected based on HapMap data (version 3 release 27) using Haploview software (Barrett et al. 2005). Selected tSNPs captured over 92% of the polymorphic variation in the region (minor allele frequency (MAF)>5% and r^2>0.8) in Caucasian population standards. Genotyping of variants was performed using a combination of TaqMan probes and Sequenom MassArray iPLEX as previously described (Traboulsee et al. 2014; Nishioka et al. 2010). Genotyping success rate was over 99.4% for all variants and without deviation from Hardy-Weinberg equilibrium expectation (p-value > 0.005). Statistical association was determined using logistic regression analysis adjusted for age and gender, in addition the combined cohort analysis was adjusted for site. Genotypes were dichotomized as presence versus absence of the minor allele (dominant model). The combined dataset was obtained by pooling samples from all populations. Segregation was quantified using non-parametric and parametric linkage analysis. Non-parametric linkage analysis were performed using SimWalk2 software (version 2.91) and NPL-All statistic (Sobel et al. 2001). Two-point parametric logarithm of odds (LOD) score were obtained with MLINK assuming a dominant model, with a fully penetrant disease and without phenocopies (Ott 1989). All MS patients were treated as affected, non-carrier individuals as healthy and unaffected mutation carriers were treated as having an unknown disease status. The deleterious allele was defined with a 0.0001 frequency and the marker-allele frequency determined empirically from genotyped individuals.

**Haplotype analysis**

Microsatellite markers spanning the *PLG* locus between D6S1633 and D6S297 were chosen to define the disease-carrying haplotype (Supplementary Table 1). All family members from those families identified with the PLG p.G420D mutation were genotyped. One primer for each pair was labeled with a fluorescent tag and PCR reactions were performed under standard conditions. PCR products were run on an ABI 3730xl (Life Technologies, Carlsbad, CA, USA), and analyzed using GeneMapper 4.0. Marker sizes were normalized to those reported
in the CEPH database and manually phased within each family.

RESULTS

To identify genes and variants of major effect on MS susceptibility, we applied exome sequencing analysis to a multi-incident family consisting of 12 individuals over three generations, with DNA available for nine family members, including six diagnosed with MS (Figure 1A). Exome analysis of II-1, II-4 and III-1 identified 47479, 46545 and 46580 variants, respectively. Of those, 25 missense variants with a MAF below 1% from public and proprietary databases of variants were identified in all three individuals (Supplementary Table 2). Segregation in additional family members identified ten variants shared amongst at least five of the six family members diagnosed with MS for whom DNA was available, and no more than one of the two unaffected blood relatives. Three of these variants were subsequently excluded as they were identified at a frequency over 1% in 366 ethnically matched controls (Supplementary Table 2). The seven remaining variants were genotyped in a multi-ethnic cohort consisting of 2160 MS patients and 886 unrelated healthy controls from Canada. Three variants (TGFBI, p.V608L (ss1467426521); SPINK13, p.C72R (ss1467426567); OR1E1, p.D96Y (ss1467426912)) appear to be private as they were not observed in any of the other samples genotyped in this study and have not been described in public databases of variants (Abecasis et al. 2012; Exome Aggregation Consortium et al. 2015). ARHGAP10, p.T518K (rs375188932), with a reported MAF of 5x10^{-5} in the ExAC database, was also not observed in any additional samples. Segregation of these four variants within the exome sequenced family is provided in supplementary figure 1. Of the remainder, SPATA18 p.P286L (rs150116592) was identified in two MS patients, UNC45B p.R776Q (rs34242925) was identified in one patient and one control, and PLG p.G420D (rs139071351) in 12 MS patients and one control.

Segregation for variants identified in SPATA18 and UNC45B did not support co-segregation with disease in additional families and were excluded from further analysis (Supplementary Figure 1). Segregation of PLG p.G420D identified the variant in 26 out of 30 family members diagnosed with MS (87%), 14 parents of MS patients (including eight obligate carriers) not known to suffer from MS, and 12 out of 30 family members not diagnosed with disease (Figure 1B-M). To quantifiably assess segregation we performed nonparametric and parametric linkage analysis for PLG p.G420D. The more conservative nonparametric score resulted in a LOD score of 1.29, whereas parametric linkage analysis resulted in a
Figure 1. Simplified pedigrees for families presenting the PLG p.G420D variant. Males are represented by squares and females by circles, the proband is indicated with an arrow head. Patients diagnosed with MS have black filled symbols and carriers of unknown clinical phenotype have grey filled symbols. Heterozygote carriers (M) and wild-type (wt) genotypes are indicated. An asterisk indicates an inferred carrier. Pedigree A was used for exome analysis, and with the exception of pedigree E, which is of Asian descent, all families are of Caucasian ancestry.
maximum LOD score of 5.48 (theta=0.05),
despite a penetrance estimate of 50%. Additional
support for a role in disease susceptibility is
provided by the level of conservation for the
glycine residue in mammals, indicating the
importance of this amino acid for protein
function (Figure 2). Haplotype analysis of PLG
p.G420D carriers between D6S1633 and
D6S297 did not identify a shared haplotype
amongst families (Supplementary Table 1), thus
suggesting that PLG p.G420D is a mutational
hotspot that has independently arisen in each
family rather than being inherited from a
common ancestor.

Clinical details were available for 17 PLG
p.G420D carriers, five males and 12 females
(Supplementary Table 3). The disease course
observed in these carriers was predominantly
consistent with relapsing-remitting MS or
secondary progressive MS with only two
patients presenting primary progressive MS. On
average, the age at onset of disease was 35.1
years (SD ± 9.1) with a disease duration of 19.9
years (SD ± 10.4). Disease severity was overall
relatively moderate, with an average expanded
disability status scale (EDSS) score of 3.92 (SD
± 2.9) and a median of 2.75.

Association analysis of PLG p.G420D was
performed in Caucasian samples from Canada
already genotyped for the identification of
additional PLG p.G420D families. This subset
consists of 2103 MS patients and 881 controls,
and resulted in a marginally significant
association with disease risk (p=0.046) and an
odds ratio (OR) of 10.19 (Table 1). In order to
validate this association we genotyped PLG
p.G420D in five independent cohorts from
Europe consisting of 12343 MS patients and
7916 healthy controls. Logistic regression
analysis corrected for age and gender identified
a similarly marginal association with disease in
the French cohort (p=0.049; OR=2.69) whereas
no association was observed for any additional
cohort (Table 1). Although the combined dataset
did not result in a significant association with
disease risk (p=0.117), with the exception of
Belgium which is the smallest set, all cohorts
resulted in OR greater than 1, indicating a higher
prevalence of PLG p.G420D in MS patients than
controls.

To assess whether common variants in PLG
lead to an increased susceptibility to develop MS, we
identified nine tSNPs spanning the entire PLG
loci and genotyped them in 2103 MS patients
and 881 controls from Canada (Supplementary
Table 4). Association analysis failed to identify
a significant association between any of the
tSNPs and susceptibility to MS (p>0.05). Since
common variants in PLG do not appear to have
an effect on MS disease risk, we assessed for the
presence of additional rare PLG substitutions in
MS patients. To this end we sequenced all PLG-
Table 1. Logistic regression analysis for PLG p.G420D (rs139071351) and risk of MS. F, female; M, male; OR, odds ratio; CI, confidence interval; NA, not applicable.

<table>
<thead>
<tr>
<th>Group</th>
<th>Gender</th>
<th>Age (mean ± SD)</th>
<th>Age at onset (mean ± SD)</th>
<th>Genotypes</th>
<th>p-value</th>
<th>OR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>M(%)</td>
<td></td>
<td></td>
<td>GA/GG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Canada</td>
<td>Controls</td>
<td>51.0</td>
<td>67.1 ± 9.8</td>
<td>1/880</td>
<td>0.046</td>
<td>10.19 (1.04-267.89)</td>
</tr>
<tr>
<td></td>
<td>MS patients</td>
<td>26.9</td>
<td>46.7 ± 11.7</td>
<td>12/2091</td>
<td></td>
<td></td>
</tr>
<tr>
<td>France</td>
<td>Controls</td>
<td>39.1</td>
<td>39.3 ± 13.1</td>
<td>4/668</td>
<td>0.049</td>
<td>2.69 (1.00-9.37)</td>
</tr>
<tr>
<td></td>
<td>MS patients</td>
<td>30.0</td>
<td>49.1 ± 11.4</td>
<td>32/2359</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spain</td>
<td>Controls</td>
<td>40.5</td>
<td>42.8 ± 12.8</td>
<td>34/3984</td>
<td>0.475</td>
<td>1.20 (0.73-1.96)</td>
</tr>
<tr>
<td></td>
<td>MS patients</td>
<td>34.8</td>
<td>44.5 ± 11.5</td>
<td>42/4246</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Germany</td>
<td>Controls</td>
<td>40.3</td>
<td>41.3 ± 16.8</td>
<td>11/2711</td>
<td>0.476</td>
<td>1.31 (0.63-2.84)</td>
</tr>
<tr>
<td></td>
<td>MS patients</td>
<td>29.2</td>
<td>40.5 ± 11.3</td>
<td>21/3712</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Belgium</td>
<td>Controls</td>
<td>47.2</td>
<td>56.2 ± 14.7</td>
<td>5/499</td>
<td>0.747</td>
<td>0.81 (0.23-3.04)</td>
</tr>
<tr>
<td></td>
<td>MS patients</td>
<td>34.0</td>
<td>48.3 ± 13.1</td>
<td>6/1000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Austria</td>
<td>MS patients</td>
<td>29.8</td>
<td>49.2 ± 12.1</td>
<td>7/918</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Combined</td>
<td>Controls</td>
<td>41.8</td>
<td>44.3 ± 15.9</td>
<td>55/8742</td>
<td>0.117</td>
<td>1.32 (0.93-1.87)</td>
</tr>
<tr>
<td></td>
<td>MS patients</td>
<td>31.0</td>
<td>45.1 ± 12.1</td>
<td>120/14326</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
coding exons in 293 familial probands from Canada, which identified 11 silent and 11 missense variants (Supplementary Table 5). Of those, nine missense variants with a MAF below 1% in at least two of three publicly available databases (1000G, ExAC or ESP) were genotyped in cases and controls from Canada (Abecasis et al. 2012; Exome Aggregation Consortium et al. 2015; Exome Sequencing Project). This analysis identified six variants (p.K38E, p.R89K, p.R261H, p.R490Q, p.A494V and p.R523W) at similar frequencies in MS patients and controls; whereas p.T200A (rs149145958), p.T500M (rs140970354) and p.A507V (rs372603134) were only identified in eight, two and one MS patient, respectively (Table 2). Despite all three variants being predicted likely damaging to protein function with a phred-scaled CADD score of 29.3, 14.4 and 18.9 for p.T200A, p.T500M and p.A507V, respectively (Kircher et al. 2014); and two of them being evolutionarily conserved (Figure 2), segregation and parametric linkage analysis, which resulted in negative LOD scores, does not support a role for these variants in disease pathogenicity (Supplementary Figure 2).

**DISCUSSION**

Exome sequencing analysis in a multi-incident family suffering from MS has nominated PLG p.G420D as a putative new risk factor for MS. Although four private missense variants cannot be conclusively excluded as a potential cause of disease in this kindred, and copy number changes were not evaluated, the identification of PLG p.G420D in twelve additional MS patients

---

**Figure 2. PLG variants and cross-species conservation.** Protein orthologs were aligned via ClustalO. Amino acid positions for PLG variants are highlighted in black. Protein orthologs with amino acid positions differing from those of the human sequence are indicated in gray. RefSeq accession numbers: *Homo sapiens* NP_000292.1, *Macaca mulatta* NP_001036540.1, *Mus musculus* NP_032903.3, *Rattus norvegicus* NP_445943.1, *Canis lupus familiaris* NP_001273889.1, *Sus scrofa* NP_001038055.1, *Bos taurus* NP_776376.1, *Myotis davidii* ELK34830.1, *Tarsius syrichta* XP_008066085.1, *Gallus gallus* XP_419618.2, *Danio rerio* AAH59801.1.
and one control from Canada suggests a role for PLG in MS susceptibility. Genotyping of additional family members from multi-incident families with PLG p.G420D resulted in positive co-segregation of the variant and disease, albeit with 50% reduced penetrance (Figure 1). Additional support for pathogenicity was sought from a large case-control cohort of MS patients from Europe; and although most populations present a higher prevalence of PLG p.G420D in MS patients than controls, a nominally significant difference was only observed in the French cohort (Table 1). A possible Acadian origin of PLG p.G420D was considered due to the marginal associations in the French and Canadian population; however, the wide geographical distribution of variant carriers from Canada and the lack of a shared ancestral haplotype (Supplementary Table 1) do not support this hypothesis. Association analysis for PLG p.G420D in the entire cohort resulted in a non-significant p-value of 0.117 and an OR of 1.32. Despite the overall lack of association observed, it is possible that carriers of the PLG p.G420D variant have an increased risk of developing MS, as suggested by the OR and initially observed familial segregation pattern. In contrast, common PLG tagging variants genotyped in this study were clearly not associated with MS risk in the Canadian population (Supplementary Table 4). This data corroborates previously described genome wide association studies which did not nominate common variants in PLG as a risk factor for MS (Beecham et al. 2013; Sawcer et al. 2011).

Sequencing of PLG in MS patients from Canada led to the identification of nine rare missense variants (Table 2). Six of which were subsequently identified at a similar frequency in MS patients and controls, suggesting they are not likely to have an effect on MS risk. Interestingly one of these variants (p.K38E, rs73015965) has been described as the cause of PLG deficiency type I when identified in homozygous or compound heterozygous form (Tefs et al. 2006). Similarly, p.R523W (rs4252129) has been associated with decreased plasma PLG levels (Ma et al. 2014). Severe PLG deficiency type I has been causally linked to ligneous conjunctivitis, a rare chronic inflammatory disease of mainly mucous membranes. Although there is no indication that heterozygous carriers are at an increased risk of developing disease (Tefs et al. 2006), PLG dysregulation could lead to an increased susceptibility to inflammatory and autoimmune diseases. In our study, three additional variants (p.T200A, p.T500M and p.A507V) not known to cause hypoplasminogenemia, were observed exclusively in MS patients. Although the allelic frequencies and segregation for rare missense PLG variants do not initially support a role in
Table 2. Case-control frequency for rare missense PLG variants identified in MS patients.

<table>
<thead>
<tr>
<th>dbSNP ID</th>
<th>Chromosome and position</th>
<th>Nucleotide change</th>
<th>Protein change</th>
<th>Minor allele frequency</th>
<th>ExAC Controls (n)</th>
<th>MS (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs73015965</td>
<td>6:161127501 A/G</td>
<td>p.K38E</td>
<td>0.003</td>
<td>0.006 (10)</td>
<td>0.007 (28)</td>
<td></td>
</tr>
<tr>
<td>rs143079629</td>
<td>6:161128812 G/A</td>
<td>p.R89K</td>
<td>0.007</td>
<td>0.010 (16)</td>
<td>0.010 (44)</td>
<td></td>
</tr>
<tr>
<td>rs149145958</td>
<td>6:161135876 A/G</td>
<td>p.T200A</td>
<td>0.001</td>
<td>0</td>
<td>0.002 (8)</td>
<td></td>
</tr>
<tr>
<td>rs4252187</td>
<td>6:161137790 G/A</td>
<td>p.R261H</td>
<td>0.003</td>
<td>0.007 (12)</td>
<td>0.005 (24)</td>
<td></td>
</tr>
<tr>
<td>rs140537724</td>
<td>6:161152807 G/A</td>
<td>p.R490Q</td>
<td>0.001</td>
<td>0.002 (3)</td>
<td>0.002 (9)</td>
<td></td>
</tr>
<tr>
<td>rs4252128</td>
<td>6:161152819 C/A</td>
<td>p.A494V</td>
<td>0.008</td>
<td>0.005 (8)</td>
<td>0.005 (20)</td>
<td></td>
</tr>
<tr>
<td>rs140970354</td>
<td>6:161152837 C/T</td>
<td>p.T500M</td>
<td>0.0002</td>
<td>0</td>
<td>0.0005 (2)</td>
<td></td>
</tr>
<tr>
<td>rs372603134</td>
<td>6:161152858 C/T</td>
<td>p.A507V</td>
<td>0.0001</td>
<td>0</td>
<td>0.0002 (1)</td>
<td></td>
</tr>
<tr>
<td>rs4252129</td>
<td>6:161152905 C/T</td>
<td>p.R523W</td>
<td>0.007</td>
<td>0.012 (19)</td>
<td>0.013 (56)</td>
<td></td>
</tr>
</tbody>
</table>

*dbSNP Build 138, bThe Exome Aggregation Consortium (ExAC) database
disease susceptibility, genotyping in additional MS patients is warranted to fully define these preliminary findings. PLG p.T200A seems of particular interest, as it was identified in eight MS patients and no controls (Table 2), it is evolutionary conserved (Figure 2), and a threonine to proline substitution at the same position has been identified in a patient with severe type I PLG deficiency (Tefs et al. 2006).

*PLG* is a plausible biological candidate for MS susceptibility as it is involved in the inflammatory response, blood-brain barrier (BBB) permeability, neuronal viability, and myelin degradation (Syrovets et al. 2012; Yao and Tsirka 2011; Chen and Strickland 1997; Cuzner and Opdenakker 1999). PLG has been shown to play a role in the immune response, with plasmin deficiency, the active form of PLG, resulting in a compromised inflammatory response in mouse brain (Hultman et al. 2014). Microglia and astrocytes are the primary mediators of inflammation in the central nervous system, and fibrin has been shown to activate their immune response by stimulating the production of inflammatory mediators including proinflammatory cytokines and reactive oxygen species, as well as act as a chemoattractant for immune cells (Syrovets et al. 2012; Hultman et al. 2014).

Genetic variants in *PLG* may also have an effect on brain inflammation by altering the BBB permeability. Plasmin alters BBB permeability by inducing morphological changes in brain astrocytes and endothelial cells through the reorganization of the actin cytoskeleton and the redistribution of tight junction proteins (Niego and Medcalf 2014; Yao and Tsirka 2011). In addition to its effects on the inflammatory response and BBB permeability, plasmin has also been shown to affect neuronal viability, including sprouting, plasticity and extracellular matrix-related neuronal death (Chen and Strickland 1997; Nakagami et al. 2000; Wu et al. 2000).

Plasmin activates highly active matrix metalloproteinases (MMPs) which are recognized as key proteases in the demyelination process. Synthetic inhibitors of MMPs have been found to ameliorate clinical symptoms and pathological signs in experimental autoimmune encephalomyelitis (EAE) animal models (Cuzner and Opdenakker 1999); and minocycline, which has several immunomodulating activities including the inhibition of MMP-9, has being successfully used in clinical trials as an add-on therapy for MS patients (Metz et al. 2009).

Despite the existence of extended families with a high incidence of MS (Fagnani et al. 2015; Sadovnick 1993), only one rare pathogenic mutations has been reported (Wang et al. 2016). In this study, the implementation of exome
sequencing analysis in a multi-incident MS family nominated PLG p.G420D as a potential susceptibility risk for MS. Additional support was provided by 10 additional multi-incident MS families in which the variant segregates with disease, albeit with reduced penetrance. Disappointingly, genotyping of PLG p.G420D in a large European case-control cohort failed to identify a significant association with MS, thus not supporting a role in disease. Despite this lack of association, dysregulation of the PLG/plasmin activation cascade is a plausible pathomechanism of MS; which in conjunction with the positive segregation of PLG p.G420D in families (Figure 1), the overall higher incidence of PLG p.G420D carriers in European MS patients (Table 1), and the identification of additional rare PLG substitutions in MS patients not observed in controls (Table 2), warrants further genetic and functional characterization of PLG in order to elucidate its potential role on MS susceptibility and pathogenesis.

ACKNOWLEDGEMENTS
We are grateful to all individuals who generously participated in this study. We thank Kevin Atkins for data collection and extraction. We also thank Généthon, AFM, ARSEP and CRB-REFGENSEP.

FUNDING
This research was undertaken thanks to funding from the Canada Research Chair [950-228408] and Canada Excellence Research Chair programs [214444], Canadian Institutes of Health Research [MOP-137051], Vancouver Coastal Health Research Institute, the Milan & Maureen Ilich Foundation [11-32095000], and the Vancouver Foundation [ADV14-1597]. Replication studies received funding from the program “Investissements d’avenir” ANR-10-IAIHU-06. Fondo de Investigación Sanitaria (FIS)-Instituto de Salud Carlos III (ISCIII)-Fondos Europeos de Desarrollo Regional (FEDER), Unión Europea [grant numbers P12/00555, PI13/01527, PI13/01466 and PI13/0879 to FM, AA and GI] and Junta de Andalucía-FEDER [grant number CTS2704 to FM]. BD is a Clinical Investigator of the Research Foundation Flanders (FWO-Vlaanderen). AG and BD are supported by the Research Fund KU Leuven (OT/11/087 and CREA/14/023) and the Research Foundation Flanders (G073415N).

AUTHORS CONTRIBUTIONS
COMPETING INTERESTS
ALT reports personal fees from Biogen Idec, Chugai, Medimmune, Teva Innovation, and EMD Serono, and grants and personal fees from Genzyme Sanofi and Roche. All other authors report no disclosures.

REFERENCES
Exome Sequencing Project, NHLBI GO Exome Sequencing Project (ESP), Seattle, WA (http://evs.gs.washington.edu/EVS/) [2014].
Nakagami, Y., K. Abe, N. Nishiyama, and N. Matsuki, 2000 Laminin degradation by


