The *CYP24A1* Gene Variant rs2762943 Is Associated With Low Serum 1,25- Dihydroxyvitamin D Levels In Multiple Sclerosis Patients

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Abstract

Background: Vitamin D is considered to play a role in multiple sclerosis (MS) etiopathogenesis. We recently identified a polymorphism located in the cytochrome P450 family 24 subfamily A member 1 (*CYP24A1*) gene, rs2762943, that was found to be associated with an increased risk for MS. *CYP24A1* codes for a protein that is involved in the catabolism of the active form of vitamin D. Here, we investigated the immunological effects of carrying the risk allele for the rs2762943 polymorphism, as well as its role as genetic modifier in MS patients.

Methods: Serum levels of 25-hydroxyvitamin D (25OHD) and 1,25-dihydroxyvitamin D (1,25(OH)₂D) were measured in a cohort of 167 MS patients. In a subgroup of these patients, expression levels of MHC class II and co-stimulatory molecules were determined by flow cytometry in blood cell populations, and the levels of proinflammatory (IFNG, GM-CSF, CXCL13) and anti-inflammatory (IL-10) cytokines and neurofilament light chain were measured by single-molecule array assays in serum samples. The effect of the rs2762943 polymorphism on disease activity and disability progression measures was evaluated in a cohort of 340 MS patients.

Results: Compared to non-carriers, MS patients carrying the risk allele for rs2762943 were characterized by reduced levels of 1,25(OH)₂D ($p=0.0001$), and elevated levels of IFNG ($p=0.03$) and GM-CSF ($p=0.008$), whereas no significant differences were observed between risk allele carriers and non-carriers groups for the other evaluated markers. The presence of the risk allele for rs2762943 had no significant impact on the annualized relapse rate, EDSS and MSSS measures during follow-up. However, risk allele carriers were younger at disease onset ($p=0.04$).

Discussion: These findings suggest that the *CYP24A1* rs2762943 gene variant plays a more important role on MS susceptibility than on disease prognosis, and is associated with lower 1,25(OH)₂D levels and heightened pro-inflammatory environment in MS patients.

Introduction

Multiple sclerosis (MS) is a complex immune-mediated disorder of the central nervous system (CNS) wherein both a polygenic background and environmental factors contribute not only to MS risk but also to disease activity [1-5]. One of the environmental factors involved in MS etiopathogenesis is vitamin D status. In recent years, numerous studies have revealed that lower serum 25-hydroxyvitamin D (25OHD) levels are associated with an increased risk for MS [4,5]. Furthermore, vitamin D status is also associated with MS disease activity, and patients with lower serum 25OHD levels were shown to have higher disease activity, though findings seem to be stronger for radiological rather than clinical outcomes [6]. Similarly, vitamin D supplementation in relapsing-remitting MS (RRMS) patients significantly improved the development of new magnetic resonance imaging lesions but had no significant effects on the annualized relapse rate or disability progression [7].

In a recent study, by means of targeted resequencing in 524 MS patients and 546 healthy controls and subsequent genotyping in an independent cohort of 3450 MS patients and 1688 healthy controls, we identified a variant located in the cytochrome P450 family 24 subfamily A member 1 (*CYP24A1*) gene, rs2762943, that was associated with an increased risk for MS [8]. The *CYP24A1* gene encodes a protein that initiates the degradation of the physiologically active form of vitamin D [1,25-dihydroxyvitamin D - 1,25(OH)₂D]. Based on these findings on *CYP24A1*, a gene involved in vitamin D metabolism, and considering the role of vitamin D in MS etiopathogenesis, as outlined above, in the present study we aimed to investigate the functional immunological effects and clinical consequences of carrying the rs2762943 risk allele in MS patients.

Materials And Methods

Quantification of serum levels of 25OHD, 1,25(OH)₂D and calcium by chemiluminescence immunoassays.

A schematic flow chart summarizing the main steps performed in study design is represented in Fig. 1. Serum 25OHD, 1,25(OH)₂D and calcium levels were measured in 167 patients with relapse-onset MS (RRMS and secondary progressive MS - SPMS). Of these, 44 (35.8%) patients were carriers of the risk minor allele for the rs2762943 polymorphism of the *CYP24A1* gene (*GT/TT*) and 123 (64.2%) patients were non-carriers (*GG*). These patients were part of a full cohort of 340 MS patients with available serum samples to measure vitamin D and calcium levels. None of the 167 MS patients were receiving disease modifying therapies (DMT) at the time of blood collection and had never received vitamin D supplements. Table 1 shows a summary of demographic and main clinical characteristics of these patients.

Briefly, peripheral blood was collected by using standard venipuncture and allowed to clot spontaneously for 30 min. Serum was isolated by centrifugation and stored frozen at -80°C until used. Serum levels of 25OHD, 1,25(OH)₂D and calcium were measured by commercially available chemiluminescence immunoassays (CLIA) according to the manufacturers' recommendations: Liaison 25 OH Vitamin D Total Assay (DiaSorin, USA) and Liason XL 1,25 Dihydroxyvitamin D (DiaSorin, USA), and a colorimetric method for Calcium Arseanzeno (Beckman Coulter, USA). Assays were run on a fully automated Liason XL analyzer (DiaSorin) for both 25OHD and 1,25(OH)₂D levels, and the AU5800 analyzer (Beckman Coulter) for calcium levels.

Immunophenotyping of peripheral blood cells and flow cytometric analysis.

Immunophenotyping was performed in peripheral blood mononuclear cells (PBMC) from a subgroup of 15 MS patients, 6 risk allele carriers for the rs2762943 polymorphism and 9 non-carriers, that were collected at the same time as the serum samples used to measure vitamin D levels (Table S1). Briefly, PBMC were isolated by Ficoll-Isopaque density gradient centrifugation (Gibco BRL, Life Technologies LTD, UK) and stored in liquid nitrogen until used. For immunophenotyping, PBMC were stained on ice for 20 min with fluorophore-conjugated antibodies against CD11c (1:25, B-ly6, BD Bioscience), HLA-DR (1:50,

L243, Biolegend), CD19 (1:100, HIB19, Biolegend), CD14 (1:50, TuK4, Thermo Fisher), CD16 (1:50, eBioCB16, Thermo Fisher), CD3 (1:50, HIT3a, Biolegend), CD56 (1:100, MEM-188, Thermo Fisher), CD86 (1:20, IT.2, Biolegend), CD40 (1:20, 5C3, Biolegend), and CD80 (1:20, 2D10, Biolegend). Cell viability was determined using Zombie Aqua (1:250, Biolegend). PBMCs were washed by centrifugation 400 x g for 5 min at 4°C to remove unbound antibodies. PBMCs were resuspended in PBS and used for flow cytometry analysis. Data acquisition was carried out using CytExpert 2.3 software and CytoFLEX flow cytometer (Beckman Coulter). FlowJo 10.6.1 was used for data analysis.

Quantification of serum biomarker levels by single-molecule array assays.

Serum levels of C-X-C motif chemokine ligand 13 (CXCL13), interferon gamma (IFNG), interleukin 10 (IL-10) and granulocyte-macrophage colony-stimulating factor (GM-CSF) were measured in 20 MS patients, 10 risk allele carriers for the rs2762943 polymorphism and 10 non-carriers (Table S1). Serum neurofilament light chain (NfL) levels were measured in 49 MS patients, 26 risk allele carriers and 23 non-carriers (Table S1). Biomarker levels were quantified in the same serum samples used to determine the vitamin D status using commercially available immunoassay kits (CXCL13 cat#102635, IFNG cat#103337, IL-10 cat#101643, GM-CSF cat#102329, and NFL cat#103186, Quanterix) and run on the fully automated ultrasensitive Simoa HD-1 Analyzer (Quanterix). Samples were run in duplicate in accordance with manufacturers' instructions with appropriate standards and internal controls. Both the mean intra-assay coefficient of variation of duplicates and the mean inter-assay coefficient of variation were <11% for all assays.

CYP24A1 rs2762943 polymorphism and disease activity and disability progression measures.

The role of rs2762943 as genetic modifier of MS was evaluated in the full cohort of 340 patients. Of these, 72 (21.2%) patients were risk allele carriers and 268 (78.8%) were non-carriers (Table 2).

In addition to demographic and main clinical characteristics such as sex, disease phenotype, disease duration, and percentage of MS patients receiving DMT, the following disability variables were recorded: Expanded Disability Status Scale (EDSS) and Multiple Sclerosis Severity Score (MSSS) at 10 years of disease duration and at year 2015. The annualized relapse rate (ARR) at 10 years from disease onset was recorded as inflammatory disease activity variable. We considered year 2015 as the maximum date of follow-up ("last follow-up time") to ensure that none of the MS patients had ever received vitamin D supplements. Based on the clinical evidence existing about vitamin D as predictor of disease activity and progression [9], from year 2016 serum vitamin D levels are routinely measured in our setting, and vitamin D supplementation is indicated for those MS patients with low vitamin D levels. Age at disease onset age was defined as the age at which patients first experienced neurologic symptoms suggestive of MS.

Statistical analysis.

Serum levels of 25OHD, 1,25(OH)₂D, calcium, cytokines and NfL, and expression of HLA class II and costimulatory molecules by blood cell subsets were compared between risk allele carriers and non-

carriers using the Mann-Whitney U nonparametric test. The effect of carrying the risk allele in age at disease onset, inflammatory disease activity and disability measures was evaluated using appropriate parametric and nonparametric tests depending on the normality of data. Analysis was performed in the whole MS population and in patients stratified according to the MS phenotype. All statistical analyses were performed using the Statistical Package for Social Sciences (IBM Corp. IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY: IBM Corp.). P-values <0.05 were considered statistically significant.

Results

CYP24A1 rs2762943 polymorphism is associated with MS and vitamin D levels.

We perused the most recent large-scale genetic study of MS susceptibility to test the association of the *CYP24A1* rs2762943 gene variant [3]. The variant had an odds ratio (OR) of 1.47 for the minor allele *T* that reached genome-wide level of statistical significance at the study's discovery phase ($p=3.9 \times 10^{-8}$; Table S2). Rs2762943 was located underneath the association peak of a nearby variant also located in the *CYP24A1* gene, rs2248137 (OR=1.12; $p=7.8 \times 10^{-11}$; Fig. S1). After controlling for the top variant, association of rs2762943 with MS remained nominally significant (OR=1.28; $p=1.2 \times 10^{-3}$; Table S2). The two variants have a large allele frequency difference, but the two risk alleles are part of the same haplotype ($D'=0.9713$; Table S3), suggesting a putative common contribution to MS susceptibility.

Next, we searched the GWAS catalog to identify any association of the *CYP24A1* rs2762943 polymorphism with other phenotypes and traits [10]. We observed genome-wide associations with metabolites and metabolite biomarkers ($p<5 \times 10^{-8}$), including vitamin D levels (Table S4), altogether suggesting that the variant is interrupting key metabolic mechanisms.

Serum 1,25(OH)₂D levels are decreased in MS patients carrying the risk allele.

Based on the abovementioned association between rs2762943 and vitamin D related traits, we first investigated whether the presence of the risk allele for the *CYP24A1* rs2762943 polymorphism was associated with changes in the serum levels of 25OHD and 1,25(OH)₂D in our cohort of 167 MS patients. As shown in Fig. 2a, the vast majority of MS patients (98.2%) had low serum 25OHD levels (<30 ng/ml) but no significant differences were observed between risk allele carriers (*GT/TT*) and non-carriers (*GG*) for rs2762943. Only 4 patients (2.4%) had serum 1,25(OH)₂D levels below the normal limit of normality (19.9 pg/ml; Fig. 2a). However, in contrast to 25OHD, MS patients carrying the risk allele for rs2762943 had significantly lower serum 1,25(OH)₂D levels compared to non-carriers ($p=0.0001$). These findings in the vitamin D status of MS patients were not associated with changes in total calcium concentration and most of the patients had serum calcium levels within the intervals of reference (8.8 - 10.6 mg/dl; Fig. 2b). As depicted in Fig. 2c, differences in serum 1,25(OH)₂D levels between risk allele carriers and non-carriers were maintained irrespective of the season of blood collection. For serum 25OHD levels, seasonal stratification did not result in statistically significant differences between risk allele carriers and non-

carriers, although a trend towards lower 25OHD levels in risk allele carriers was observed ($p=0.06$) in blood samples collected in cold season.

Expression of the MHC class II and co-stimulatory markers in blood cell populations is similar between risk allele carriers and non-carriers.

We next investigated whether risk allele carriers for the rs2762943 polymorphism differed from non-carriers in terms of steady state myeloid and lymphoid cell activation profiles. For this, we assessed the expression levels of MHC class II and co-stimulatory molecules on classical monocytes and B cells. As shown in Fig. 3, the presence of the risk allele was not associated with changes in the expression levels for HLA-DR, CD40, CD80, and CD86. Similarly, in T cells, the risk allele was not associated with changes in the expression levels of HLA-DR.

Serum levels of the proinflammatory cytokines IFNG and GM-CSF are increased in risk allele carriers.

As a next step, we evaluated whether the presence of the risk allele for the rs2762943 polymorphism was associated with changes in the levels of pro-inflammatory (CXCL13, IFNG, GM-CSF) and anti-inflammatory (IL-10) cytokines. As shown in Fig. 4a, serum levels of IFNG and GM-CSF were significantly higher in risk carriers compared to non-carriers ($p=0.03$ and $p=0.008$ respectively), whereas serum levels of CXCL13 and IL-10 were similarly distributed between both groups of patients.

Presence of the CYP24A1 rs2762943 risk allele has no impact on MS disease activity and disability progression measures but influences age at disease onset.

Main demographic and clinical characteristics such as sex, MS phenotype, disease duration and percentage of patients who received DMT at any time during follow-up were comparable between risk allele carriers and non-carriers (Table 2).

When the rs2762943 polymorphism was evaluated as genetic modifier of MS, we observed that risk allele carriers were younger at disease onset compared to non-carriers ($p=0.04$; Table 3). Comparison of disability measures such as EDSS and MSSS both at 10 years from disease onset and at last follow-up did not reveal significant differences between risk allele carriers and non-carriers (Table 3). Similarly, ARR at 10 years from disease onset was comparable between both groups of patients (Table 3). Further stratification of the whole MS group into disease phenotypes (RR, SP, and primary progressive) was not associated with significant differences for age at disease onset, inflammatory disease activity and disability measures between risk allele carriers and non-carriers (Table S5).

Based on the findings of increased levels of pro-inflammatory cytokines in risk allele carriers, and in view of the strong associations reported in the literature between NfL and radiological measures of disease activity such as the number of contrast-enhancing lesions and number of T2 lesions [11], serum levels of NfL were also measured in a subgroup of MS patients as a proxy of CNS radiological inflammation. Fig. 4b shows the distribution of serum NfL levels in risk allele carriers and non-carriers, which were comparable among both groups of patients.

Discussion

In the present study, we investigated the functional immunological consequences of carrying the risk allele for the rs2762943 polymorphism in the *CYP24A1* gene, as well as its role as genetic modifier in MS patients. This gene variant, which is located 672 base pairs upstream from the transcriptional start site of the *CYP24A1* gene [10], was found to be associated with an increased risk for MS in a recent targeted resequencing study carried out by our group [8] and also in the largest GWAS up to now conducted in MS [2]. Interestingly, rs2762943 remains as an independent signal for association with MS and is linked to vitamin D related traits [10].

CYP24A1 encodes a mitochondrial P450 enzyme that can hydroxylate both 25OHD and 1,25(OH)₂D producing the inactive metabolites 24,25(OH)₂D and 1,24,25(OH)₃D, respectively [12,13]. In consequence, CYP24A1 limits the amount of the active form of vitamin D in target tissues by speeding up its catabolism and also by reducing the pool of 25OHD available for 1 α -hydroxylation in the kidney [12, 13]. Although it can hydroxylate both, 1,25(OH)₂D is the preferred substrate for CYP24A1 [14]. In our study, MS patients carrying the risk allele for rs2762943 (T) had significantly reduced serum levels of 1,25(OH)₂D compared to non-allele carriers. Of note, the lower 1,25(OH)₂D levels in risk allele carriers were not associated with significantly reduced serum 25OHD levels in these patients, despite that 25OHD levels were overall low in most MS patients. Although not proved, these findings may be consistent with an increased enzymatic activity for CYP24A1 that results in an accelerated catabolism of the active form of vitamin D in those MS patients carrying the risk allele for rs2762943.

Although the lower 1,25(OH)₂D levels observed in risk allele carriers were not of sufficient magnitude to alter the calcium concentration, inasmuch as serum calcium levels were within the normal range and similar to non-carriers, we further explored the potential immunological effects associated with reduced serum 1,25(OH)₂D levels in these patients. Investigation of the expression levels for HLA class II and co-stimulatory molecules such as CD40, CD80, and CD86 in the major PBMC populations did not reveal significant differences between risk allele carriers and non-carriers. In contrast, levels of the proinflammatory cytokines IFNG and GM-CSF were found to be significantly elevated in carriers of the risk allele for rs2762943. Considering that vitamin D has immunomodulatory properties and has shown to suppress Th1 and Th17 responses [15,16], the findings of increased IFNG and GM-CSF levels in MS patients carrying the risk allele may be associated with heightened Th1 and Th17 immune responses as a result of the lower blood 1,25(OH)₂D levels observed in these patients.

In a second part of the study, we evaluated whether the presence of the risk allele for the rs2762943 polymorphism in the *CYP24A1* gene could act as a genetic modifier in MS. Low vitamin D is considered a moderate risk factor for MS susceptibility based on a number of observational studies demonstrating an association between low serum 25OHD levels and increased MS risk [17-19]. The role of vitamin D influencing disease prognosis in MS is more controversial. Higher 25OHD levels have been associated with lower relapse risk [20,21], lower risk of subsequent development of new T2 lesions and contrast-

enhancing lesions on brain MRI [21], and lower change in EDSS scores [22]. However, other studies have not reported an association of vitamin D levels with MS disease activity [23]. Furthermore, a number of randomized clinical trials indicate that vitamin D supplementation does not seem to have beneficial effects on annualized relapse rate or EDSS scores in MS patients [24]. In our study, the presence of the risk allele for rs2762943 in MS patients had no effect on disease course, since it was not associated with increased inflammatory disease activity evaluated by the ARR or higher risk of disability progression evaluated by the EDSS or MSSS during follow-up. A limitation of the study was the evaluation of abovementioned inflammatory disease activity and disability progression measures at defined moments in time such as 10 years from disease onset and at year 2015 to avoid the potential confounding effect of vitamin D supplements administered to MS patients in these clinical parameters. Another limitation of the study was the lack of MRI parameters to evaluate radiological disease activity. In order to circumvent this limitation, we measured serum NfL levels in a subgroup of MS patients as a proxy of CNS radiological inflammation. Although it cannot totally ruled out, the finding of similar NfL levels between risk allele carriers and non-carriers does not support the presence of significant differences in radiological measures of disease activity such as the number of T2 lesions or the number of contrast-enhancing lesions between both groups of patients. Noteworthy, risk allele carriers were younger at disease onset compared to non-carriers, indicating that the *CYP24A1* rs2762943 gene variant seems to have more impact on MS susceptibility rather than on disease prognosis.

Conclusion

Altogether, these findings indicate that the risk allele for the rs2762943 polymorphism in the *CYP24A1* gene is associated with lower serum 1,25(OH)₂D levels and heightened pro-inflammatory environment in MS. Additional studies are needed to further investigate the mechanisms by which the presence of the risk allele for the *CYP24A1* rs2762943 gene variant is associated with an increased risk for MS.

Abbreviations

1,25(OH)₂D: 1,25-dihydroxyvitamin D

25OHD: 25-hydroxyvitamin D

ARR: Annualized relapse rate

CLIA: Chemiluminescence immunoassays

CXCL13: C-X-C motif chemokine ligand 13

CYP24A1: cytochrome P450 family 24 subfamily A member 1

DMT: Disease modifying therapies

EDSS: Expanded Disability Status Scale

GM-CSF: Granulocyte-macrophage colony-stimulating factor

IFNG: Interferon gamma

IL-10: Interleukin 10

MS: Multiple sclerosis

MSSS: Multiple Sclerosis Severity Score

NfL: Neurofilament light chain

OR: Odds ratio

PBMC: Peripheral blood mononuclear cells

RRMS: relapsing-remitting multiple sclerosis

Declarations

Acknowledgment

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- Consent for publication

Not applicable.

- Availability of data and materials

The data that support the findings of this study are available from corresponding author on reasonable request.

- Competing interests

The authors declare that they have no competing interests.

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- Authors' contributions

S.M. and L.M.: Drafting/revision of the manuscript for content, including medical writing for content; Major role in the acquisition of data; Analysis or interpretation of data. O.C.: Major role in the acquisition

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All authors reviewed the manuscript.

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Tables

Table 1. Summary of demographic and baseline clinical characteristics of the MS patients used to measure serum 25OHD, 1,25(OH)₂D and calcium levels.

Baseline characteristics	Risk allele carriers (<i>GT/TT</i>)	Non-carriers (<i>GG</i>)
N (%)	44 (35.8%)	123 (64.2%)
Age (years)	38.4 (10.3)	37.5 (11.2)
Female/Male (% Females)	29/15 (65.9%)	80/43 (65.0%)
Disease duration (years)¹	6.1 (6.5)	5.4 (6.1)
EDSS^{a,2}	2.0 (1.5-3.0)	2.0 (1.5-3.0)
Season of blood collection^b		
Spring & summer	19 (43.2%)	52 (42.3%)
Autumn & winter	25 (56.8%)	71 (57.7%)

Data are expressed as mean (standard deviation) unless otherwise stated. ^aData are expressed as median (interquartile range). ^bRefers to season when blood was collected to measure 25OHD, 1,25(OH)₂D and calcium levels. ¹Disease duration was calculated as the difference between time of blood collection and disease onset. ²Refers to EDSS scores at the time of blood collection. EDSS: Expanded Disability Status Scale.

Table 2. Main demographic and clinical characteristics of the full cohort of MS patients used to evaluate the effect of the *CYP24A1* rs2762943 gene variant and disease activity and disability progression.

Variables	Risk allele carriers (<i>GT/TT</i>)	Non-carriers (<i>GG</i>)	P value
N (%)	72 (21.2%)	268 (78.8%)	-
Female/Male (% Females)	45/27 (62.5%)	182/86 (67.9%)	0.38
MS phenotype			
RR (N %)	28 (38.9)	92 (34.3)	
SP (N %)	29 (40.3)	86 (32.1)	0.16
PP (N %)	15 (20.8)	90 (33.6)	
Disease duration (years)¹	20 (6.8)	20.4 (6.8)	0.67
% of patients treated²	54/72 (75%)	195/260 (75%)	1.00

Data are expressed as mean (standard deviation) unless otherwise stated. ¹Disease duration was calculated as the difference between the time of last follow-up (year 2015) and disease onset. ²Refers to the percentage of patients that received disease modifying therapies at any moment from disease onset to the time of last follow-up (year 2015). RR: relapsing-remitting; SP: secondary progressive; PP: primary progressive.

Table 3. Association of CYP24A1 rs2248137 polymorphism with age at disease onset, and disease activity and disability progression measures.

Variables	Risk allele carriers (<i>GT/TT</i>)	Non-carriers (<i>GG</i>)	P value
Age at disease onset (years)	29.3 (10.1)	32.3 (11.3)	0.04
	(N=72)	(N=264)	
Disability measures			
EDSS at 10 years^a	3.0 (1.8 - 4.2)	3.5 (2.0 - 6.0)	0.23
	(N=58)	(N=191)	
MSSS at 10 years^a	3.8 (2.1 - 5.5)	4.5 (2.3 - 7.4)	0.23
	(N=58)	(N=191)	
EDSS at last follow-up^{a,1}	4.0 (2.0 - 6.5)	5.5 (2.5 - 7.0)	0.24
	(N=58)	(N=202)	
MSSS at last follow-up^{a,1}	3.0 (1.6 - 6.6)	4.5 (1.6 - 6.9)	0.34
	(N=54)	(N=184)	
Inflammatory measure			
ARR at 10 years	3.4 (3.4)	2.7 (2.8)	0.14
	(N=50)	(N=202)	

Data are expressed as mean (standard deviation) unless otherwise stated. ^aData are expressed as median (interquartile range). ¹Last follow-up time corresponds to year 2015. ARR: annualized relapse rate. EDSS: Expanded Disability Status Scale. MSSS: Multiple Sclerosis Severity Score. The number of MS patients available for each clinical comparison is shown in parentheses).

Supplemental Data

Supplemental figure 1 is not available with this version.

Figures

Figure 1

Flowchart showing the study design. The effect of the rs2762943 polymorphism located in the *CYP24A1* gene was investigated in a cohort of 340 MS patients. In a subcohort of 167 patients with available serum samples, levels of 25-hydroxyvitamin D (25OHD), 1,25-dihydroxyvitamin D (1,25(OH)₂D) and calcium were measured by chemiluminescence immunoassays. The immunological effects of the rs2762943 polymorphism were investigated in 3 subgroups of MS patients with blood samples collected at the same time as the serum samples used to measure vitamin D levels: (i) Immunophenotyping was performed by flow cytometry in PBMC (peripheral blood mononuclear cells) from 15 MS patients to determine the expression of HLA class II and costimulatory molecules; (ii) Levels of pro-inflammatory and anti-inflammatory cytokines were measured by Simoa (single-molecule array) assays in serum samples from 20 MS patients; (iii) Levels of neurofilament light chain (NfL) were quantified by Simoa in serum samples from 49 MS patients. Finally, the role of rs2762943 as genetic modifier was investigated in the full cohort of 340 MS patients by analyzing the association between the polymorphism and the EDSS (Expanded Disability Status Scale), MSSS (Multiple Sclerosis Severity Score), annualized relapse rate (ARR), and age at disease onset.

Figure 2

Comparisons of serum 1,25(OH)₂D, 25OHD and calcium levels between MS patients carrying the risk allele for the rs2762943 polymorphism of the *CYP24A1* gene (GT/TT) and non-carriers (GG). (a) Box plots showing levels of 25OHD in ng/ml and 1,25(OH)₂D in pg/ml. (b) Boxplot showing calcium levels in mg/dl. (c) Box plots showing 1,25(OH)₂D levels in MS patients segregated according to the season of blood collection. Dashed lines indicate the lower limit of normality for 25OHD and 1,25(OH)₂D, and the interval of reference for serum calcium levels. Number of patients included in each group and determination is shown in parentheses.

Figure 3

Box plots showing comparisons of expression levels of MHC class II (HLA-DR) and co-stimulatory molecules (CD80, CD86, CD40) in peripheral blood B cells, T cells, and classical monocytes in risk allele carriers (*GT/TT*) and non-carriers (*GG*). Number of patients included in each comparison is shown in parentheses. MFI: Median fluorescence intensity.

Figure 4

Comparisons of serum levels of cytokines and NfL in risk allele carriers (*GT/TT*) and non-carriers (*GG*). Box plots showing levels of pro-inflammatory (CXCL13, IFNG and GM-CSF) and anti-inflammatory (IL-10) cytokines in pg/ml (a). Box plot showing NfL levels in pg/ml (b). Number of patients included in each comparison is shown in parentheses.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [Supplementarymaterial.doc](#)