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# Interleukin-10 promoter polymorphism in multiple sclerosis: association with disease progression

## Abstract

Interleukin-10 (IL-10) is a potent anti-inflammatory and immunosuppressive cytokine that modulates disease expression in multiple sclerosis (MS) and experimental autoimmune encephalomyelitis (EAE). In previous studies, two dinucleotide repeat elements (microsatellites G and R) were identified, respectively located about 1.1 and 4.0 kb upstream of the IL-10 gene transcription initiation site. Several of their alleles were found to be associated with the level of IL-10 production. The aim of our study was to determine whether sequence

variations in the IL-10 gene were associated with MS susceptibility and progression. To do so, we analyzed the distribution of IL-10.R and IL-10.G alleles and genotype polymorphisms in MS patients and healthy controls. We then correlated our findings with disease severity in MS patients using the progression index (PI). Patients were classified as experiencing mild (PI < 0.5) or severe (PI > 0.5) disease progression. Our results show no association between the IL-10.R microsatellite and MS, regardless of disease severity. However, IL-10.G microsatellite genotyping showed that IL-10.G9/9, G10/13, G11/13 and G13/14 were more frequently found in patients with mild disease progression ($p = 0.005$). We also found that in patients with severe disease progression, IL-10.G9/10, G9/11, G9/13 and G12/13 were over-represented ($p = 0.002$). Our study indicates that neither the IL-10.R or the IL-10.G alleles are associated with predisposition to MS. However, several IL-10.G genotypes might emerge as markers of disease progression.

## Full text

### INTRODUCTION

Multiple sclerosis (MS) is a chronic inflammatory, demyelinating disease of the central nervous system involving autoimmune mechanisms [1]. MS is characterized by plaques of demyelination and axonal disruption in the brain and spinal cord. Although the disease may be triggered by specific immune recognition processes primarily involving T lymphocyte subsets and monocytes, the mechanisms initiating the inflammatory reaction in MS lesions remain unknown [2]. However, deviation of the regulatory balance of pro- and anti-inflammatory cytokines production has been related to disease susceptibility and clinical expression [3, 4]. There is now evidence that both cell-cell contacts and cytokines, such as transforming growth factor (TGFbeta-) and interleukin (IL)-10, play a major role in the regulation of the immune response, in particular in protective mechanisms of regulatory T cells against autoimmune diseases [5]. An increased production of proinflammatory cytokines such as interferon gamma (IFN-gamma), IL-2 and tumor necrosis factor-alpha (TNF-alpha) has been reported in MS brain lesions [6, 7]. In contrast, beneficial effects on experimental autoimmune encephalo myelitis (EAE) have been associated with the polarisation of the T helper 2 (Th2) response (IL-4, IL-10 and TGF-beta production) [8]. IL-10 is mainly produced by Th2 lymphocytes and monocytes/macrophages. Its function is central to the regulation of the

inflammatory response: it downregulates the production of proinflammatory cytokines (IL-1, IL-6, TNF-alpha and IFN-gamma), and reduces the cell surface expression of immune receptors such as major histocompatibility complex (MHC) class II [9-11]. The immunosuppressive functions of IL-10 have been extensively studied in MS. Some authors have reported that IL-10 mRNA levels and disease activity were inversely correlated [12, 13].

Genetic factors are likely to be involved in MS risk determination [14-16]. Some data also indicate that although the MHC region plays a significant role in MS susceptibility, much of the genetic effects in MS remain to be explained [17]. Polymorphism has been described in the promoter of human IL-10 DNA [18-20]. Recently, it was demonstrated that the production of IL-10 is under genetic control [21], and that haplotypes associating microsatellites IL-10.R2/IL-10.G14 and IL-10.R3/IL-10.G7 were associated with high and low IL-10 secretion, respectively [22]. Pickard *et al.* did not find any association between the three single nucleotide polymorphisms (position -1082, -819, -592) and susceptibility to, or severity of MS [23]. Others have demonstrated that there was no association between IL-10 mRNA expression and the different polymorphisms [24]. In our study, we analyzed the distribution of IL10.G and IL-10.R microsatellites located approximately 1.1 and 4.0 kb upstream of the IL-10 gene transcription site in MS patients and normal controls. Our objective was to investigate whether IL-10 polymorphism was associated with MS or could modify clinical aspects of the disease.

## PATIENTS AND METHODS

### *Patients and controls*

The patients group included 153 unrelated and unselected patients with clinically defined MS, recruited after approval by the local ethics committee. Written informed consent was obtained from all patients. Patients were all Northern European Caucasians and were treated in the department of Neurology (CHU of Lille, France). Patients had clinically defined MS for relapsing-remitting and secondary progressive forms [25], and defined or probable MS for primary progressive MS [26]. Disability was scored using the Kurtzke Expanded Disability Status Scale (EDSS) [27]. As between and *intra* observer EDSS variations are important, clinical evaluation was performed by only two neurologists (JDS, PV). Furthermore, the same patient was always evaluated by

the same neurologist. One hundred and fifteen patients had the relapsing remitting form and 38 patients had the progressive forms (16 secondary progressive and 22 primary progressive according to the Lublin *et al.* criteria) [28]. The rate of accumulation of neurological disability was evaluated with the Progression Index (PI = EDSS/duration [years]), in order to reflect the course of disease more accurately. Both EDSS and PI evaluations were performed at least three months after a relapse. MS patients were stratified for severity of disease outcome [29]. Eighty-six patients were classified as having mild disease progression (PI < 0.5), and 67 as having severe disease progression (PI > 0.5) [30]. A random sample of 184 ethnically-matched, healthy subjects was selected from the same region. The demographic and clinical data describing patients and controls are presented in Table 1.

### *IL-10.R and IL-10.G microsatellite genotyping*

Peripheral blood mononuclear cells (PBMC) from patients and healthy volunteers were purified from heparinized whole blood by Ficoll-Hypaque density gradient centrifugation (Pharmacia-Amersham, Uppsala, Sweden). DNA was extracted from PBMC using the Wizard® DNA purification system (Promega®, Madison, USA) according to the manufacturer's instructions.

The IL-10.R microsatellite was genotyped (n = 170 (91 healthy controls and 79 MS patients)) using the following forward primer labeled with 6-carboxyfluorescein (6-FAM 5'-CCCTCCAAAATCTATTTGCATAAG-3') and a reverse primer (5'-CTCCGCCCAGTAAGTTTCATCAC-3'). After an initial melting time of 7 min at 95° C, samples were subjected to 30 rounds of 94° C 1min, 61° C 1min, 72° C 1 min, and a final extension time of 5 min at 72° C.

Genotyping at the IL-10.G microsatellite (n = 337 (184 healthy controls and 153 MS patients)) was performed using the following forward primer labeled with 6-carboxyfluorescein (6-FAM 5'-GTCCTTCCCCAGGTAGAGCAACACTCC-3') and a reverse primer (5'-CTCCCAAAGAAGCCTTAGTAGTGTTG-3'). After an initial heating time of 7 min at 95° C, samples were subjected to 30 cycles of 94° C 1 min, 65° C 1 min, 72° C 1 min, and a final extension time of 5 min at 72° C.

The GeneAmp PCR sytem 9700 (Perkin Elmer, Courtaboeuf, France) was used in each case. For both IL-10.R and IL-10.G microsatellites, besides the genomic DNA (1 mug), each reaction contained sense and antisense primers (0.1 mug/mul

each), 1U of Taq polymerase (Perkin Elmer), 200 muM of each of the 4dNTP, 1X PCR buffer (Perkin Elmer) supplemented with 2.5 mM $MgCl_2$. The final reaction volume was 40 mul. One mul of the PCR products was mixed with formamide, loading buffer E and 0.5 mul of ROX-500 standard size (PE Applied Biosystems), heated to 95° C for 5 min and then cooled immediately on ice, prior to separation on a sequencing gel containing 5% acrylamide and 6M urea at 3000V, 60mA, 200W and 51° C on a 377XL DNA sequencer (Perkin Elmer), usually for 3 hours.

Alleles were designated as previously described: 1-4 for IL-10.R microsatellite and 1-16 for the IL-10.G microsatellite [18, 19].

### *Statistical methods*

Allele distributions in the control and patient groups were compared using the Chi-square test for multiple independent variables. The degree of freedom was equal to one less than the number of alleles included in the analysis. When alleles occurred relatively rarely ($n < 8$), frequencies were combined, as small expected numbers would not degrade the accuracy of the chi-square test. We considered that a p-value below 0.05 as a significant difference between groups. The IL-10.R analysis included only 79 patients and 91 normal subjects due to the low number of alleles. In order to determine if the number of subjects was sufficient, confidence intervals for the differences were computed.

A Chi-square test was used to analyze the IL-10.R genotype.

We used a multiple correspondence analysis in order to determine the IL-10.G genotype distribution among MS subgroups. With the multiple correspondence analysis, we were able to associate the relevant genotypes with disease severity and secondarily use a chi-square test [31]. We also performed a logistic regression in order to evaluate the different relative risks.

## RESULTS

### *IL-10.R Alleles in MS patients and controls*

We identified four alleles named IL-10.R1 to IL-10.R4 (Table 2). We observed that IL-10.R2 (controls 64.2% (117/182), MS 71.5% (113/158) and IL-10.R3 (controls 31.3% (57/182), MS 27.2% (43/158) alleles accounted for the majority of IL-10.R

polymorphism. IL-10.R allele distributions did not reveal any significant difference between controls and MS subjects ($khi^2$ = 1.05, degree of freedom = 1, p = 0.305). Likewise there was no difference between subgroups (controls *versus* mild progression $khi^2$ = 1.148, df = 1, p = 0.284; controls *versus* severe progression $khi^2$ = 0.272, df = 1, p = 0.602; mild progression *versus* severe progression $khi^2$ = 0.155, df = 1, p = 0.694). The differences and the confidence intervals for the comparison between control and MS groups were 7.2% [- 2.6; 17.1] for IL-10.R2 and 4.1% [- 13.8; 5.6] for IL-10.R3.

### *IL-10.R genotypes in MS patients and controls*

We did not find any difference in genotype frequencies between MS patients and healthy individuals ($khi^2$ = 1.492, df = 1, p = 0.222) or between subgroups (controls *versus* mild progression $khi^2$ = 1.835, df = 1, p = 0.176; controls *versus* severe progression $khi^2$ = 0.292, df = 1, p = 0.586; mild progression *versus* severe progression $khi^2$ = 0.360, df = 1, p = 0.549).

### *IL-10.G alleles in MS patients and controls*

IL-10.G microsatellite was highly polymorphic, generating 10 alleles in our population of individuals. These were equally distributed between MS patients and healthy controls ($khi^2$ = 2.043, df = 7, p = 0.957; Table 3). Figure 1 shows allele frequencies in the control group and in each subgroup of MS patients (with mild and severe progression). There was no difference in the allele frequency of IL-10.G in the MS subgroups compared with controls when all the alleles were considered (controls *versus* mild progression $khi^2$ = 7.116, df = 7, p = 0.417; controls *versus* severe progression $khi^2$ = 3.293, df = 7, p = 0.857; mild progression *versus* severe progression $khi^2$ = 11.224, df = 7, p = 0.129). However, significant differences were noted between mild and severe MS progression for IL-10.G7 ($khi^2$ = 4.796, df = 1, p = 0.029) and IL-10.G12 ($khi^2$ = 5.593, df = 1, p = 0.018). Table 3 shows the frequency of IL-10.G alleles between PPMS and patients with bout-onset MS which was equally distributed between these two subgroups.

### *IL-10.G genotypes in MS patients and controls*

The IL-10.G marker proved to be quite polymorphic and highly heterozygous, generating 38 genotypes in our population. Several of them being very poorly

represented, we selected 12 genotypes encompassing 78.5% (265/337) of subjects for the genotype analysis. The multiple correspondence analysis indicated that four genotypes were associated with each form of MS. Chi square analysis defined statistical significances of these associations. Genotypes IL-10.G 9/9, G10/13, G11/13 and G13/14 were significantly associated with mild MS progression ($khi^2$ = 8.057, df = 1, p = 0.005), whereas IL-10.G 9/10, G9/11, G9/13 and G12/13 genotypes were significantly associated with severe MS progression ($khi^2$ = 9.166, df = 1, p = 0.002) (Figure 2). Logistic regression showed that these genotypes were associated with each subgroups of MS with an odds ratio of 2.1 [0.98; 4.46] and 0.5 [0.2; 1.14] for severe and mild progression respectively. Although, confidence limits included "1", these results confirmed the tendency of an association between each group of genotype and MS progression. We did not note any significant difference in the other genotypes. Furthermore, we did not find an association between alleles and genotypes with sex or age at onset.

## DISCUSSION

Our allele analysis failed to establish any relevant relationship between IL-10.R and IL-10.G microsatellite distributions and MS susceptibility. However, IL-10.G genotypes were distributed differently in MS patients depending on the rate of their disease progression.

Epidemiological studies have established that susceptibility to MS is influenced by genetic factors [32, 33]. The genetic component in MS may be due to several genes, in particular in the MHC region [14-16], but the incomplete penetrance of MS susceptibility alleles probably results from interactions with other genes and post-transcriptional regulatory mechanisms. Genome screening has failed to find a major susceptibility locus, in particular in the 1q31-32 region containing the IL-10 gene [34]. However, the presence of polymorphism within non-structural parts of a relevant gene (such as the promoter region or other regulatory elements) might affect expression levels [35, 36]. It was recently demonstrated that the production of IL-10 was under genetic control [21]. Such polymorphism may be a useful chromosomal marker in genetic studies of MS and other immunological diseases, as recently reported in systemic lupus erythematosus [37, 38]. A previous study failed to demonstrate any association between the three single nucleotide polymorphisms (position -1082, -819, -592) of IL-10 promoter, and susceptibility to MS [23]. Nevertheless, high and low IL-10 secretion levels have been linked to several haplotypes associating different

microsatellites [22]. Our results did not demonstrate any significant difference in the alleles and genotypes frequency of IL-10.R between MS groups compared with healthy subjects. We confirm that the previously reported CA repeat is not very polymorphic [19]. In our study, IL-10.R2 and IL-10.R3 accounted for the majority of alleles found in 98.7% of MS patients (156/158) or 95.6% of controls (174/182). As there are only four alleles described in the literature [19], we analysed a smaller number of subjects for IL-10.R than IL-10.G polymorphisms. The lack of difference between controls and MS with regard to IL-10.R2 and IL-10.R3 was confirmed by the logistic procedure.

Our data also failed to detect any significant difference in the IL-10.G allele distributions between MS patients and controls. Similar results were reported in MS by McDonnell *et al.* [39]. However, we did observe that IL-10.G7 is correlated with mild MS progression, while IL-10.G12 is correlated with severe MS progression. In addition, previous studies have shown that the IL-10.G7 allele is associated with low IL-10 secretion [22]. These results are surprising, but should not be over-interpreted, since IL-10G.7 and IL-10G.12 are rare alleles in Northern European Caucasians (less than 10%).

IL-10.G being highly polymorphic, we used a multiple correspondence analysis. Results showed that several genotypes were associated with MS progression. We found that IL-10.G9/9, G10/13, G11/13 and G13/14 genotypes were associated with mild MS progression. In contrast, IL-10.G9/10, G9/11, G9/13 and G12/13 were associated with severe MS progression. In our population, these genotypes were found in over 64% (98/153) of MS patients. These alleles, except for IL-10.G12 and IL-10.G14, were indifferently distributed in the 2 groups of genotypes, and were associated respectively with mild and severe MS, suggesting a complex regulatory mechanism in the production of IL-10. However, some alleles such as IL-10.G14 have been associated with a high IL-10 production [22]. It may prove interesting to investigate a possible correlation between IL-10 genotypes, IL-10 levels, and clinical features in MS patients. However, it is actually difficult to assess reliable secretion of IL-10 by *in vivo* or *in vitro* assays. First, several biases have been described for measurement of cytokines by ELISA or functional *in vitro* assays [40]. Moreover, variation in protein expression levels has recently been attributed to post-transcriptionnal mechanisms [41]. Powel *et al.* [41] reported that the 3'-untranslated region of IL-10 mRNA presents multiple copies of potential IL-10 mRNA destabilizing motifs, making mRNA analysis difficult [41].

Our results failed to associate any particular IL-10.R or IL-10.G alleles with the progression index of MS. However, we did find an association between some IL-10.G genotypes and MS progression. Kube *et al.* have reported that the sequence located between -1100/-900 base pairs of the transcription initiation site, including microsatellite IL-10.G, regulates IL-10 expression [42]. The ability to control this immunoregulatory cytokine might have a crucial effect on the initiation and progression of the disease. Finding a link between an IL-10 gene and disease progression could also help to predict disease outcome in newly diagnosed individuals. Since several treatments, such as INF-beta, affect IL-10 secretion [43, 44], we suggest that some genotypes may be associated with better therapeutic response. Other sequential studies in patients with long term therapeutic follow-up are needed to confirm this hypothesis.

CONCLUSION

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## Figures

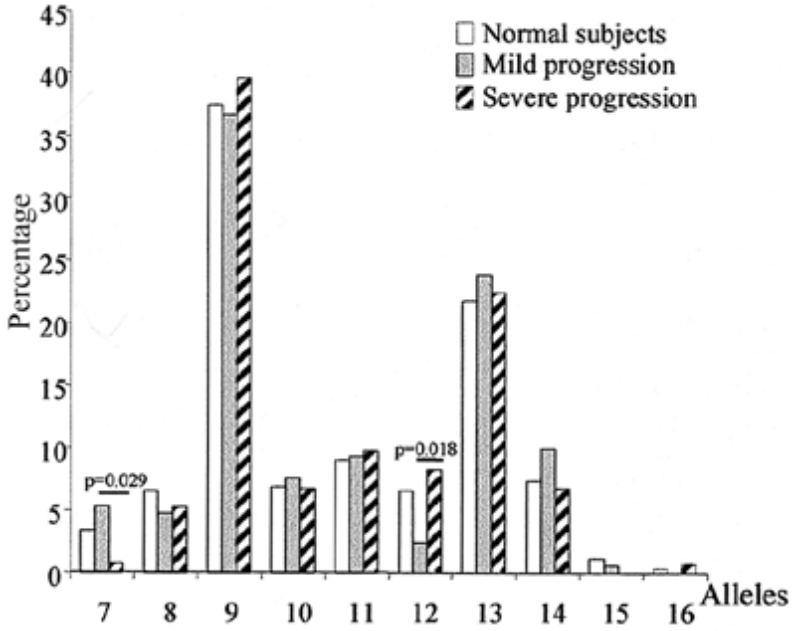


Figure 1

**Allele distribution of the IL-10.G microsatellite marker in controls and MS subgroups.**

The frequency of each allele was expressed as a percentage of the total number of alleles. The statistical analysis (Chi-square test) indicates significant differences between MS subgroups for the alleles IL-10.G7 and IL-10.G12.

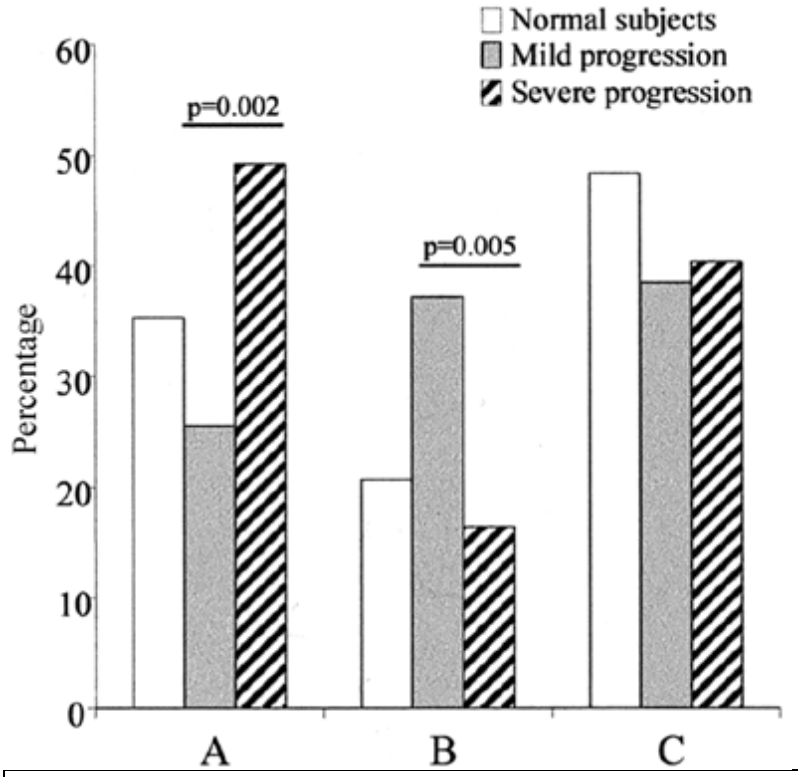



Figure 2

**Genotypes associated with MS subgroups after a multiple correspondence statistical analysis.**

**A:** subjects with one of the genotypes 9/10, 9/11, 9/13 or 12/13; **B:** subjects with one of the genotypes 9/9, 10/13, 11/13 or 13/14; C: subjects with other genotypes. The

frequency of each group of genotype was expressed as a percentage of the total number of genotypes. The statistical analysis (Chi-square test) indicated significant differences between MS subgroups.

**Table 1**
**Clinical and demographic data on patients included in the study[a]**

| | Age (years) | Sex (M/F) | EDSS | Duration of disease (years) | Progression index |
|---|---|---|---|---|---|
| All MS patients (n = 153) | 39.5 ± 10.8 (16-69) | 48/105 | 3.5 ± 2.3 (0-8.5) | 6.4 ± 4.4 (1-25) | 0.73 ± 0.67 (0-6) |
| Mild MS progression (n = 86) | 36.6 ± 9.6 (20-60) | 18/68 | 2 ± 1.3 (0-5) | 7.6 ± 5.1 (1-24) | 0.25 ± 0.14 (0-0.5) |
| Severe MS progression (n = 67) | 43.3 ± 11.0 (16-69) | 30/37 | 5.5 ± 1.5 (2-8.5) | 4.7 ± 2.5 (1-18) | 1.35 ± 0.55 (0.85-3.5) |
| Controls (n = 184) | 29.9 ± 7.0 (18-62) | 77/107 | / | / | / |

[a] All numbers are expressed as mean ± SD (range).

**Table 2**
**Allele frequencies in patients and controls for IL-10.R microsatellite marker**

| Allele[a] | Normal (n = 182) | | MS (n = 158) | | Mild progression (n = 92) | | Severe progression (n = 66) | |
|---|---|---|---|---|---|---|---|---|
| | n | % | n | % | n | % | n | % |
| R1 | 2 | 1.1 | 0 | 0 | 0 | 0 | 0 | 0 |
| R2 | 117 | 64.3 | 113 | 71.5 | 67 | 72.8 | 46 | 69.7 |
| R3 | 57 | 31.3 | 43 | 27.2 | 24 | 26.1 | 19 | 28.8 |
| R4 | 6 | 3.3 | 2 | 1.3 | 1 | 1.1 | 1 | 1.5 |

[a] as previously described by Eskdale *et al.*, 1996.
MS: multiple sclerosis; n: number of alleles.
No significant difference are observed between MS patients and healthy individuals or between subgroups.

**Table 3**
**Allele frequencies in patients and controls for IL-10.G microsatellite marker**

| Allele* | Normal (n = 368) | | MS (n = 306) | | Mild progression (n = 172) | | Severe progression (n = 134) | | RR/SPMS (n = 262) | | PPMS (n = 44) | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | n | % | n | % | n | % | No. | % | n | % | n | % |
| G7 | 12 | 3.3 | 10 | 3.3 | 9 | 5.2 | *1 | 0.7 | 10 | 3.8 | 0 | 0 |
| G8 | 24 | 6.5 | 15 | 4.9 | 8 | 4.6 | 7 | 5.2 | 12 | 4.6 | 3 | 6.8 |
| G9 | 138 | 37.5 | 116 | 37.9 | 63 | 36.6 | 53 | 39.5 | 99 | 37.8 | 17 | 38.6 |
| G10 | 25 | 6.8 | 22 | 7.2 | 13 | 7.5 | 9 | 6.7 | 18 | 6.9 | 4 | 9.1 |
| G11 | 33 | 8.9 | 29 | 9.5 | 16 | 9.3 | 13 | 9.7 | 27 | 10.3 | 2 | 4.5 |
| G12 | 24 | 6.5 | 15 | 4.9 | 4 | 2.3 | **11 | 8.2 | 11 | 4.2 | 4 | 9.1 |
| G13 | 80 | 21.7 | 71 | 23.2 | 41 | 23.8 | 30 | 22.4 | 63 | 24.0 | 8 | 18.2 |
| G14 | 27 | 7.3 | 26 | 8.4 | 17 | 9.9 | 9 | 6.7 | 21 | 8.0 | 5 | 11.4 |
| G15 | 4 | 1.1 | 1 | 0.3 | 1 | 0.6 | 0 | 0 | 1 | 0.4 | 0 | 0 |
| G16 | 1 | 0.3 | 1 | 0.3 | 0 | 0 | 1 | 0.7 | 0 | 0 | 1 | 2.3 |

* as previously described by Eskdale and Gallagher, 1995.
MS: multiple sclerosis; RR: remitting-relapsing; SP: secondary progressive; PP: primary progressive; n: number of alleles.
Significant differences are indicated between mild and severe MS progression for IL-10.G7.
(* p = 0.029) and IL-10.G12 (** p = 0.018).

## Authors

L. Almeras, B. Meresse, J. de Seze, D. Lefranc, Sylvain Dubucquoi, I. Fajardy, P. Vermersch, L. Prin

Department of Immunology, Research Center-Medical school, 59045 Lille Cedex, France.

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# Thioredoxin specifically cross-desensitizes monocytes to MCP-1

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**Les éditions JLE**

30 rue Berthollet, Bât. A,
94110 Arcueil, France

+ 33 (0)1 46 73 06 60

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