Ruolo delle Selectine nello sviluppo e nell'evoluzione della Sclerosi Multipla:

P-selectin glycoprotein ligand-1 variable number of tandem repeats (VNTR) polymorphism in patients with Multiple Sclerosis

E-selectin *A561C* and *G98T* polymorphisms in patients with Multiple Sclerosis: association analysis and correlation with clinical data

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Introduction

Multiple Sclerosis (MS) is a chronic inflammatory disease of the Central Nervous System (CNS) in which myelin proteins are supposed to act as autoantigens. The initial step of MS pathogenesis is the aberrant activation of specific populations of autoreactive T lymphocytes in the periphery, followed by T cell recruitment into the brain (for review, see[1]). The recruitment of T lymphocytes represents a critical event in the pathogenesis of the disease. The process leading to lymphocyte extravasation is a regulated sequence of steps controlled by both adhesion molecules and activating factors. It involves: 1) initial contact (tethering) and rolling along the vessel wall mediated by selectins and integrins; 2) chemoattractant-induced intracellular molecular changes leading to integrin activation; 3) integrin-dependent firm arrest; 4) diapedesis[2]. So far, it has been shown that both E- and P-selectins are required for efficient tethering and rolling of neutrophils in brain vessels in mice treated with TNF-α[3]. Selectins are a family of cell-surface glycoproteins: E-, P-, and L-selectin. All selectins consist of a lectin-like N-terminal domain, an epidermal growth factor (EGF)-like domain, a variable number of short consensus repeats, a transmembrane domain and a cytoplasmic domain (for review, see[4]). E- and P-selectin are expressed by inflamed cerebral endothelium and bind T

lymphocytes bearing the cutaneous lymphocyte antigen (CLA). CLA acts as the principal human T lymphocyte ligand for E-selectin[5,6] and has been shown to be expressed as a post-translational modification of P-selectin glycoprotein ligand-1 (PSGL-1)[7]. E-selectin molecules recruit lymphocytes of the Th1 subtype, which produce proinflammatory cytokines and possess the CLA antigen, while do not support binding of Th2 lymphocytes, in which PSGL-1 is expressed but not modified post-transcriptionally[8,9]. Recently, Piccio et al.[2] demonstrated that antibodies (Abs) against PSGL-1 and anti E- and P-selectin block tethering and rolling of autoreactive lymphocytes, further supporting the hypothesis that PSGL-1/endothelial selectins are critical in the recruitment of lymphocytes in inflamed brain venules[2].

Multiple Sclerosis is a multifactorial disease, and genetic factors play a primary role in orchestrating the pathological events and in changing the disease phenotype from patient to patient. Gene polymorphisms in adhesion molecules such as selectins may act as susceptibility factors, increasing the risk of disease development, or may operate as regulatory factors, modulating the severity of pathogenic processes or the response to drug treatment. In this regard, several polymorphisms have been described within the E-selectin gene. A Single Nucleotide Polymorphism (SNP) in the coding region of the gene (*A561C*) causes a conservative change of a serine with an arginine at codon 128 (S128R), and occurs in Caucasians at a frequency of about 10%[10,11]. This mutation is located in the EGF-like domain and is particularly interesting as its presence confers an alteration in selectin ligand binding specificity such that mutant binding is no longer dependent upon the presence an α1-3-linked fucose moiety for high affinity, calcium dependent binding[12].

Consistent with this evidence, it has been demonstrated that the S128R SNP leads to a gain of function under flow conditions, implying that this mechanism may amplify the number of leukocytes that roll and subsequently arrest on endothelium[13,14]. Since the S128R mutant phenotype has been reported to be associated with an increase in frequency, early onset, and severity of atherosclerotic disease in affected individuals[15,16], it is conceivable that the enhanced binding capacity might be responsible for the disease phenotype (Revelle). Moreover, Rao et al.[17] showed that the presence of the SNP confers a different specificity in lymphocyte recruitment, as S128R E-selectin molecules are able to bind CLA-negative lymphocytes, suggesting that also non Th-1 subsets (Th2 and Th0) may be recruited. The above mentioned consideration might in part explain the observed association between the S128R and several disorders with an inflammatory component, including atherosclerosis[15,16], severe Coronary Artery Disease (CAD)[18-20] and Systemic Lupus Erythematosus (SLE)[11].

A further SNP in the E-selectin gene, consisting in a G to T mutation (G98T), occurs with a frequency of about 10% in Caucasian population[15,21]. It is located in the 5'-untranslated region of the E-selectin gene, and possibly affects its expression level[15]. Wenzel et al.[16] noticed that the S128R mutation was highly correlated with the G98T polymorphism, but more recent findings[21] didn't confirm this association, although the possibility that the G98T mutation drives the expression of the S128R mutated E-selectin cannot be excluded.

On the basis of these studies, underlying the possible importance of allelic variants in E-selectin in the pathogenesis of diseases characterized by an inflammatory component, including MS, the

distribution of the *A561C* and of the *G98T* SNPs was analyzed in a population of MS patients as well as in a same-size population of age-matched healthy subjects, in order to determine whether their presence could influence the susceptibility or exert a protective effect on the development of the disease. Besides, a possible influence of the polymorphisms on clinical outcome was analyzed, correlating the presence of the SNPs with age at onset, disease duration, time between the first and the second attack, relapse rate, progression index and ranked severity score.

Materials and Methods

Subjects

From January 2002 and December 2003, 307 patients with MS were consecutively recruited at the MS Centers of: Ospedale Maggiore Policlinico of Milan, Department of Neurology, University of Novara, and …….Bergamo. All patients underwent a standard battery of examinations, including medical history, physical and neurological examination, screening laboratory test, brain Magnetic Resonance Imaging (MRI). Diagnosis was made in accordance to McDonald 's criteria[22]. The course of MS was described as Relapsing Remitting (RR, n=229), Secondary Progressive (SP, n=34) or Primary Progressive (PP, n=20)[23]. 29 out of 229 patients with RR-MS had a benign form, defined as patients with MS for 10 years or more, who had an Expanded Disability Status Scale (EDSS) score[24] of 2 or less[25]. The remaining 24 patients had a Clinical Isolated Syndrome (CIS) suggestive of MS, and, at MRI, dissemination of lesions in time and in space[22]. The clinical data collected were: age at onset of MS, disease duration, time between the first and the second attack, number of relapses, disease severity according to EDSS. An attack refers to an episode of neurological disturbance, reported either by subjective report or by objective observation, with a duration of more than 24 hours preceded by a relatively stable or improving neurological state of at least 30 days[22]. The annual relapse rate prior to any immunomodulatory therapy was calculated in 91 patients with RR-MS having a disease duration of 2 years or more. MS progression was defined by calculating the Progression Index (PI) as a measure of accumulated disability over time (PI = EDSS/disease duration in years)[26]. PI before the beginning of any immunomodulatory treatment, was calculated in 194 patients. For patients with a disease duration of more than 5 years (n=88), the ranked severity score (EDSS/disease duration of MS within cohorts of 5 years' duration) was also calculated, in order to minimize the effect of the denominator[26].

The control group consisted of 300 subjects matched for ethnic background, gender and age. An informed consent to participate in this study was given by all individuals. All the demographic and clinical variables of subjects are summarized in Table 1.

Genomic DNA isolation

High-molecular weight DNA was isolated from whole blood using a Flexigene Kit (Qiagen, Hildren, Gemany) as described by the manufacturer.

G98T and A561C polymorphism determination

G98T and *A561C* SNPs were determined by Polymerase Chain Reaction-Restriction Fragment Length Polymorphisms (PCR-RFLP) assay.

G98T: genomic DNA was amplified using specific primers, yielding a 332 bp fragment, and then digested with *Hph*I. The transversion $G_{98} \rightarrow T$ abolishes the *Hph*I recognition site. Fragments have been visualized on an agarose gel, as previously described[21].

A561C: DNA was amplified using specific primers, yielding a 186 bp fragment, and then digested with *Pst*I. The presence of the SNP abolishes the *Pst*I recognition site. Fragments have been visualized on an agarose gel, as previously described[20].

Statistical analysis

Allelic and genotypic frequencies were obtained by direct counting. Hardy Weinberg equilibrium was tested by using a χ^2 goodness of fit test. Fisher's exact test was used to test for differences in allele distribution between the groups. Age at onset, disease duration, time between the first and the second attack, relapse rate, progression index and ranked severity score are expressed as mean ± S.E.M. Non parametric Wilcoxon rank sum test incorporating the Bonferroni correction for multiple testing was used for comparisons among

groups.

The odds ratio (OR) was calculated along with its 95% CI.

Results

The *G98T* and *A561C* allele and genotype frequencies for the study groups of MS patients and controls are reported in Tables 2 and 3, respectively. Both MS and control populations were in Hardy-Weinberg equilibrium, and the distribution of the two polymorphism in controls was similar to the previously reported one observed in Caucasians[10,11,15,21]. A trend towards an increase in the allelic frequency of the *G98T* SNP in MS patients compared with controls was observed (17.3 vs 13.8%; Table 3). Indeed, these results derive from a significantly increased number of individuals with the *T/T* genotype in MS population as compared with

healthy subjects (4.7 vs 1.0%; *P*=0.01; OR: 4.7, 95%CI: 1.3-16.6; Table 2). Stratifying MS patients according to the course of the disease[23], a marked increase in allelic frequency of the *G98T* SNP in RR-MS patients was shown as compared with progressive MS, either SP or PP (18.6 vs 11.8 and 12.5%, respectively, Table 2). Again, this increase derives from a significantly increased number of individuals with the *T/T* genotype in RR-MS population (5.2%; *P*=0.006, RR-MS vs controls; OR: 5.5, 95%CI: 1.5-19.6). Moreover, none of the patients with benign MS (more than 10 years' duration and EDSS≤2[25], n=29) was a carrier of the *T/T* genotype as compared with RR-MS patients with the same duration but EDSS>2 (n=27; Table 4).

No significant differences in the distribution of the *A561C* allelic frequency between MS patients and controls were shown (14.6 vs 13.0 %; Tab. 3). Considering MS subtypes, a decreased frequency of the *C* mutated allele was observed in SP-MS (8.8%). As the mean conversion time to SP-MS was 10.9 years, we compared SP-MS with RR-MS patients who did not progress to SP-MS after more than 11 years from disease onset (n=59). The frequency of the mutated allele in this group was higher than in SP-MS patients (14.4 vs 8.8%, *P*>0.05, Table 5), raising the possibility of a protective effect of the *C* allele towards the progressive course of the disease. In accordance with this hypothesis, the mean time occurred before the progression to SP subtype was 17.0 years in *C* carriers (n=5) as compared with 10.8 years in non carriers (n=29). Stratifying patients by gender, no statistically significant differences in either allele or genotype distribution were observed both for *G98T* and *A561*C SNPs (data not shown). None of the possible haplotypes deriving from the combination of the two alleles was associated with MS (Table 6), although the *TTCC* genotype was absent in MS population.

Concerning clinical variables, no significant differences were found with regard to disease duration and age at onset between patients carrying the *T* or *C* mutated alleles, or both, as compared with non carriers (Table 7). Interestingly, the presence of the *CC* genotype resulted associated with an earlier mean age at onset than non-carriers (20.2 vs 31.7 years, n=6, $P>0.05$). Stratifying by the presence of the mutated alleles, no differences on the time occurred between the first and the second attack (n=158) and on the relapse rate in RR-MS patients with more than 2 years' disease duration were found (Table 7). No effects of the polymorphisms on the progression of MS, evaluated with PI and ranked severity score, were shown as well (Table 7).

A statistically significant correlation between ranked severity score and relapse rate in RR-MS patients was found ($\rho=0.39$; $P=0.007$; n=47, data not shown).

Discussion

According to the results shown, the presence of the *T/T* genotype of the *G98T* SNP in the E-selectin gene confers an increased risk to develop MS. Moreover, it seems to be a risk factor towards the RR subtype and, among RR-MS patients, a complete absence of this genotype has been observed in benign RR-MS, raising the possibility of an influence of the *T/T* genotype on the evolution of the disease. On the contrary, the *A561C* polymorphism does not increase the risk of MS, but is likely to act as protective factor towards the progression to SP-MS in subjects affected by RR-MS. Despite these genetic findings strongly support an association between the mutations studied and MS, no significant differences were found with regard to the clinical variables considered.

The polymorphisms studied are located within a gene which is crucial for recruitment of activated lymphocytes into the brain[4], thus an association of the *T/T* genotype of the *G98T* SNP with the RR subtype, characterized by attacks of symptomatic inflammatory demyelination, in which production of intrathecal inflammatory molecules is marked[27], could be reasonable. The observation that the frequency of the *G98T* mutated allele is not increased in progressive forms, (both PP and SP), characterized by a low degree of inflammation and a consistent axonal injury[28], further supports this hypothesis.

At present, the molecular mechanism underlying this association is still unknown. The *G98T* SNP is located in the 5'-untranslated region of the E-selectin gene, thus an effect on this selectin expression, such as an increase in mRNA transcription, is conceivable, as also previously suggested[21]. In accordance with previous findings[21], we did not find a direct correlation between *G98T* and *A561C* mutations in our populations. Interestingly, none of MS patients carried the *TTCC* haplotype, but the number of subjects analyzed is too small to reach significant conclusions.

The *A561C* SNP has been demonstrated to exert a profound effect on ligand recognition and binding, as cell transfectants expressing the *A561C* E-selectin better support interactions with neutrophils under flow conditions[13]. This tethering mechanism could amplify the number of lymphocytes interacting with mutated cerebral endothelial cells during demyelinating attacks occurring in RR-MS. In this regard, analysis of relapse rate in our patients did not show differences between *C* carriers and non carriers. On the other hand, the mutated *C* allele could influence the severity rather than the number of relapses. This is a very important issue to consider, as the wild type E-selectin recruits specifically activated Th-1 lymphocytes[8], which produce proinflammatory cytokines and chemokines[1]. On the contrary, the presence of the *A561C*

polymorphism extends the range of lymphocytes recruited by E-selectin, including Th2 lymphocytes[17]. This subset of T cells produce anti-inflammatory cytokines, which could help to counterbalance the negative effects of proinflammatory Th1 cells. With regards to RR-MS patients, the presence of the *C* allele could be beneficial, allowing the recruitment of anti-inflammatory Th2 cells besides inflammatory Th1 lymphocytes. The observed decreased frequency of the *A561C* SNP in SP-MS and the longer time to conversion in SP in *C* carriers are consistent with this hypothesis, suggesting a protective effect on the progression of the disease, although we were not able to directly correlate the presence of the mutation with the disability, measured with the PI and the ranked severity score. Indeed, MS is considered a disease with both a genetic and environmental component, thus it should be taken into account a possible interaction between these and other allelic variants all over the genome, mainly in other adhesion molecules implicated in the migration of lymphocytes, as well as with possible non genetic factors. Analyses on clinical variables available failed to demonstrate any significant association with the presence of the polymorphisms, but, interestingly, a positive correlation between the relapse rate and the ranked severity score in RR-MS patients not treated with immunomodulatory drugs was evidenced, suggesting that acute exacerbations have a sustained effect on disability, as also previously shown[23].

To conclude, the *T/T* genotype of the *G98T* SNP could be proposed as risk factor for RR-MS, while the *A561C* is likely to exert a protective effect towards the progression to SP. Although any risk haplotype has been identified, the number of patients having the *T/T* risk factor and the mutated C carrier was very low, thus analysis on larger populations is certainly needed. Besides, the possible effect of the combination of the two alleles needs further investigations on in-vitro as well as an in-vivo models.

References

1. Galimberti D, Bresolin N, Scarpini E. Chemokine network in multiple sclerosis: role in pathogenesis and targeting for future treatments. Expert Rev Neurotherapeutics 2004; 4(3): 439-453.

2. Piccio L, Rossi B, Scarpini E, et al. Molecular mechanisms involved in lymphocyte recruitment in inflamed brain microvessels: critical roles for P-selectin glycoprotein ligand-1 and heterotrimeric Gi-linked receptors. J Immunol 2002; 168; 1940-1949.

3. Carvalho-Tavares J, Hickey MJ, Hutchinson J, et al. A role for platelets and endothelial selectins in TNFα-induced leukocyte recruitment in the brain microvasculature. Circ Res 2000; 87(12): 1141-1148.

4. Ley K. The role of selectins in inflammation and disease. Trends Mol Med 2003; 9(6): 263-268.

5. Lenter M, Levinovitz A, Isenmann S, Vestweber D. Monospecific and common glycoprotein ligands for E- and P-selectin on myeloid cells. J Cell Biol 1994; 125(2): 471-481.

6. Li F, Wilkins PP, Crawley S, et al. Post-translational modifications of recombinant P-selectin glycoprotein ligand-1 required for binding to P- and E-selectin. J Biol Chem 1996; 271(6): 3255-3264.

7. Fuhlbrigge RC, Kieffer JD, Armerding D, Kupper TS. Cutaneous lymphocyte antigen is a specialized form of PSGL-1 expressed on skin-homing T cells. Nature 1997; 389(6654): 978-981.

8. Austrup F, Vestweber D, Borges E, et al. P- and E-selectin mediate recruitment of T-helper-1 but not T-helper-2 cells into inflamed tissues. Nature 1997; 385: 81-83.

9. Borges E, Tietz W, Steegmaier M, et al. P-selectin glycoprotein ligand-1 (PSGL-1) on T helper 1 but not on T helper 2 cells binds to P-selectin and supports migration into inflamed skin. J Exp Med 1997; 185: 573-578.

10. Wenzel K, Hanke R, Speer A. Polymorphism in the human E-selectin gene detected by PCR-SSCP. Hum Genet 1994; 94: 319-330.

11. El-Magadmi M, Alansari A, Teh LS, et al. Association of the A561C E-selectin polymorphism with Systemic Lupus Erythematosus in 2 independent populations. J Rheumatol 2001; 28: 2650-2652.

12. Revelle BM, Scott D, Beck PJ. Single amino acid residues in the E- and P-selectin epidermal growth factor domains can determine carbohydrate binding specificity. J Biol Chem 1996; 271: 16160-16170.

13. Rao RM, Clarke JL, Ortlepp S, et al. The S128R polymorphism of E-selectin mediates neuraminidase-resistant tethering of myeloid cells under shear flow. Eur J Immunol 2002; 32: 251-260.

14. Yoshida M, Takano Y, Sasaoka T, et al. E-selectin polymorphism associated with myocardial infarction causes enhanced leukocyte-endothelial interactions under flow conditions. Arterioscler Thromb Vasc Biol 2003; 23: 783-788.

15. Wenzel K, Felix S, Kleber FX, et al. E-selectin polymorphism and atherosclerosis: an association study. Hum Mol Genet 1994; 3: 1935-1937.15.

16. Wenzel K, Ernst M, Rohde K, et al. DNA polymorphisms in adhesion molecule genes-a new risk factor for early atherosclerosis. Hum Genet 1996; 97: 15-20.

17. Rao RM, Haskard DO, Landis CR. Enhanced recruitment of Th2 and CLA-negative lymphocytes by the S128R polymorphism of E-selectin. J Immunology 2002; 169: 5860-5865.

18. Wenzel K, Blackburn A, Ernst M, et al. Relationship of polymorphisms in the renin-angiotensin system and in E-selectin of patients with early severe coronary heart disease. J Mol Med 1997; 75: 57-61.

19. Ye SQ, Usher D, Virgil D, et al. A *Pst*I polymorphism detects the mutation of Serine 128 to Arginine in CD 62E gene – A risk factor for Coronary Artery Disease. J Biomed Sci 1999; 6: 18-21.

20. Ellsworth DL, Bielak LF, Turner ST, et al. Gender- and age-dependent relationships between the E-selectin S128R polymorphism and coronary artery calcification. J Mol Med 2001; 79: 390-398.

21. Zheng F, Chevalier JA, Zhang LQ, et al. An HphI polymorphism in the E-selectin gene is associated with premature coronary artery disease. Clinical Genetics 2001; 59: 58-64.

22. McDonald IW, Compston A, Edan G, et al. Recommended diagnostic criteria for MS. Ann Neurol 2001; 50: 121-127.

23. Lublin FD, Reingold SC, for the National Multiple Sclerosis Society (USA) Advisory Committee on clinical trials of new agents in Multiple Sclerosis. Defining the clinical course of multiple sclerosis; results of an international survey. Neurology 1996; 46(4): 907-911.

24. Kurtzke JF. Rating neurological impairment in multiple sclerosis: an expanded disability status scale (EDSS). Neurology 1983; 33: 1444-1452.

25. Pittock SJ, Mayr WT, Jorgensen NW, et al. Clinical implications of benign multiple sclerosis: a 20-year population-based follow-up study. Ann Neurol 2004; 56: 303-306.

26. Fazekas F, Strasser-Fuchs S, Kollegger H, et al. Apolipoprotein E ε4 is associated with rapid progression of multiple sclerosis. Neurology 2001; 57: 853-857.

27. Scarpini E, Galimberti D, Baron PL, et al. IP-10 and MCP-1 levels in CSF and serum from multiple sclerosis patients with different clinical subtypes of the disease. J Neurol Sci 2002; 195: 41-46.

28. Bitsch A, Schuchardt J, Bunkowski S, et al. Acute axonal injury in multiple sclerosis. Correlation with demyelination and inflammation. Brain 2000; 123: 1174-1183.

P-selectin glycoprotein ligand-1 variable number of tandem repeats (VNTR) polymorphism in patients with Multiple Sclerosis

Text

The initial step of multiple sclerosis (MS) pathogenesis is the aberrant activation of specific populations of autoreactive T lymphocytes in the periphery, followed by T cell recruitment into the brain (for review see [5]). The process leading to lymphocyte extravasation is a finely regulated sequence of steps controlled by adhesion molecules [12], including P-selectin glycoprotein ligand-1 (PSGL-1) on lymphocytes. PSGL-1 gene is located on chromosome 12q24 [19] and several polymorphisms have been described within its sequence. Among them, a variable number of tandem repeat polymorphism (VNTR) in the mucin-like region originates three possible allelic variant, named alleles *A*, *B* and *C*, from the largest containing 16 decameric repeats, to the smallest, consisting of 14 tandem repeats, respectively [1]. The allelic frequency of this polymorphism in Caucasians is 85% *A*, 14% *B* and 1% *C*,and an association of the smaller *B* and *C* alleles with a lower risk of developing acute cerebrovascular (CVD) events has been demonstrated [8]. From a functional point of view, recent findings demonstrate that activated platelets bind less efficiently to neutrophils carrying the shortest *C* allele.

Therefore, the protective association of *B* and *C* alleles with CVD make it conceivable that binding of leukocytes to activated platelets or endothelium may vary among alleles differing in size [8], as also previously suggested by transfection studies pointing out that distance from plasma membrane might be an important determinant of P-selectin binding activity [14]. Further association analyses excluded any association of the shorter alleles with coronary heart disease [4, 18], although the VNTR and M62I polymoprhisms in PSGL-1 independently influence its plasma levels [18].

On the basis of these studies, the distribution of the VNTR polymorphism was analyzed in a population of MS patients as well as in a same-size population of age-matched healthy subjects, in order to determine whether the presence of different alleles could influence the susceptibility or the course of the disease.

Three hundred twenty one patients with MS were consecutively recruited at MS Centers of Ospedale Maggiore Policlinico, Milan and Ospedale Maggiore, Novara. All patients underwent a standard battery of examinations, including medical history, physical and neurological examination, screening laboratory test, brain Magnetic Resonance Imaging (MRI). Diagnosis was made in accordance to McDonald 's criteria [11]. The course of MS was described as Relapsing Remitting (RR, n=241), Secondary Progressive (SP, n=37) or Primary Progressive (PP, n=21) [9]. The remaining 22 patients had a Clinical Isolated Syndrome (CIS) suggestive of MS, and, at MRI, dissemination of lesions in time and in space [11]. The clinical data collected were: age at onset of MS, disease duration, time between first and second attack, number of relapses, disease severity according to the Expanded Disability Status Scale (EDSS) [6], annual relapse rate and disease progression before treatment (Table 1). The control group consisted of 342 subjects matched for ethnic background, gender and age. An informed consent to participate in this study was given by all individuals.

High-molecular weight DNA was isolated from whole blood using a Flexigene Kit (Qiagen, Hildren, Gemany) as described by the manufacturer.

VNTR distribution was determined by Polymerase Chain Reaction (PCR) assay, as previously described [1].

Allelic and genotypic frequencies were obtained by direct counting. Hardy Weinberg equilibrium was tested by using a χ^2 goodness of fit test. Fisher's exact test was used for differences in allele distribution between the groups, and the odds ratio (OR) was calculated along with its 95% CI. Clinical data of subjects carrying different genotypes were compared using the Mann-Whitney *U*-test. Statistical significance was set at $P<0.05$.

The VNTR allele and genotype frequencies for the study groups of MS patients and controls are reported in Table 2. The distribution of the three possible alleles *A*, *B* and *C* in controls was similar to the previously reported one observed in Caucasians [4]. No significant differences between allelic frequencies of the three alleles in MS as compared with controls were shown (A: 81.5 versus 84.1%; B: 16.6 versus 15.0%; C: 1.9 versus 0.9%, P>0.05, Table 2). However, stratifying patients according to the course of the disease [9], a significant increased frequency of the shortest *C* allele in PP-MS either comparing with controls (7.1 versus 0.9% , P=0.011, OR: 9.3, 95%CI: 2.2-40.4) or with all the remaining MS patients, who had acute inflammatory attacks at onset and an initial RR form (7.1 versus 1.5%, P=0.036, OR: 5.4, 95%CI: 1.3-21.7). Besides, none of SP-MS patients was a carrier of the *C* allele and *B* carriers converted later from RR to SP course as compared with *A/A* subjects (15.8 versus 8.8 years, P=0.01).

Stratifying patients by gender, no statistically significant differences were observed. No further correlations between genetic findings and clinical variables described in Table 1 were observed.

According to the present results, the presence of the *C* allele of the VNTR polymorphism might increase the susceptibility to develop PP-MS, a type of MS which is a progressive form from the onset without any relapses or remissions, rather than the most common form of MS in which attacks of symptomatic demyelination and subsequent recovery occur. In addition, this allelic variant is absent in SP-MS patients studied and the presence of the shorter *B* allele is likely to delay the transition from RR to SP course.

The polymorphism studied is located within a gene which is crucial for the recruitment of activated lymphocytes into the brain [7]. From a biological point of view, the presence of the *C* allele has been demonstrated to exert a profound effect on ligand binding, as cell transfectants expressing the shortest *C* allele support less efficiently interactions with endothelium [8]. Thus, the distance from plasma membrane is likely to be a crucial factor influencing P-selectin binding activity. Basing on these considerations, it could be conceivable that the *A* allele confers to lymphocytes the highest efficiency to bind P-selectin during acute attacks. Therefore, carriers of the shorter *B* or *C* alleles could have a less efficient recruitment of lymphocytes into the brain, implying a less severe demyelination during exacerbations. This mechanism could result over time in a slower progression of the disease to the SP form, a condition in which axonal damage is the prevalent cause of the accumulating disability. In this regard, previous data already demonstrated that acute exacerbations have a measurable and sustained effect on disability in patients with RR-MS [10]. The absence of the *C* allele in SP-MS patients analyzed, together with the observed longer time before SP conversion in *B* carriers, are consistent with the previous hypothesis.

The high frequency of *C* carriers found in PP-MS patients suggests that an efficient recruitment of lymphocytes is not a crucial step during the pathogenesis of this form, which is supposed to have a lower degree of inflammation and a consistent axonal damage [3, 16]. Although PP and SP-MS have been considered subtypes of a chronic progressive form of MS,

controversy remains with regard to the nature of PP-MS as to whether it is a distinct disease. In fact, several features are different in PP-MS as compared with bout-onset MS, including brain MRI , which could be normal [17], absent oligoclonal bands in cerebrospinal fluid [13], less perivascular cuffing and parenchymal cellular infiltration [15] and different laboratory parameters [2].

In conclusion, the *C* allele of the VNTR polymorphism in PSGL-1 gene is likely to be associated with PP-MS. Besides, data in SP-MS suggest that the longer the allele, the better the efficiency to bind P-selectin, possibly influencing the course of bout-onset MS. However, confirmation studies on larger groups are needed, in order to perform a functional analysis, possibly in carriers of the rare *C/C* genotype. Besides, a possible interaction between these and other allelic variants all over the genome, mainly in other adhesion molecules implicated in the migration of lymphocytes should be considered.

References

[1] V. Afshar-Kharghan, R. Diz-Küçükkaya, E.H. Ludwig, A.J. Marian, J.A. López, Human polymorphism of P-selectin glycoprotein ligand 1 attributable to variable numbers of tandem decameric repeats in the mucinlike region, Blood 97 (2001) 3306-3307.

[2] K. Bashir, J. Whitaker, Clinical and laboratory features of primary progressive and secondary progressive MS, Neurology 53 (1999) 765-771.

[3] A. Bitsch, J. Schuchardt, S. Bunkowski, T. Kuhlmann, W. Bruck, Acute axonal injury in multiple sclerosis. Correlation with demyelination and inflammation, Brain 123 (2000) 1174-1183.

[4] P. Bugert, M.M. Hoffmann, B.R. Winkelmann, M. Vosberg, J. Jahn, M. Entelmann, H.A. Katus, W. März, U. Mansmann, B.O. Boehm, S. Goerg, H. Klüter, The variable number of tandem repeat polymorphism in the P-selectin glycoprotein ligand-1 gene is not associated with coronary heart disease, J. Mol. Med. 81 (2003) 495-501.

[5] D. Galimberti, N. Bresolin, E. Scarpini, Chemokine network in multiple sclerosis: role in pathogenesis and targeting for future treatments, Expert Rev. Neurotherapeutics 4 (2004) 439-453.

[6] J.F. Kurtzke, Rating neurological impairment in multiple sclerosis: an expanded disability status scale (EDSS), Neurology 33 (1983) 1444-1452.

[7] K. Ley, The role of selectins in inflammation and disease, Trends Mol. Med. 9 (2003) 263-268.

[8] M.L. Lozano, R.C. Conejero, J. Corral, J. Rivera, J.A. Iniesta, C. Martinez, V. Vicente, Polymorphisms of P-selectin glycoprotein ligand-1 are associated with neutrophil-platelet adhesion and with ischaemic cerebrovascular disease, British J. Haematol. 115 (2001) 969-976.

[9] F.D. Lublin, S.C. Reingold, for the National Multiple Sclerosis Society (USA) Advisory Committee on clinical trials of new agents in Multiple Sclerosis, Defining the clinical course of multiple sclerosis; results of an international survey. Neurology 46 (1996) 907-911.

[10] F.D. Lublin, M. Baier, G. Cutter, Effect of relapses on development of residual deficit in multiple sclerosis, Neurology 61 (2003) 1528-1576.

[11] W.I. McDonald, A. Compston, G. Edan, D. Goodkin, H.P. Hartung, F.D. Lublin, H.F. McFarland, D.W. Paty, C.H. Polman, S.C. Reingold, M. Sandberg-Wollheim, W. Sibley, A. Thompson, S. van den Noort ,B.Y. Weinshenker, J.S. Wolinsky, Recommended diagnostic criteria for MS, Ann.Neurol. 50 (2001) 121-127.

[12] L. Piccio, B. Rossi, E. Scarpini, C. Laudanna, C. Giagulli, A.C. Issekutz, D. Vestweber, E.C. Butcher, G. Constantin, Molecular mechanisms involved in lymphocyte recruitment in inflamed brain microvessels: critical roles for P-selectin glycoprotein ligand-1 and heterotrimeric Gi-linked receptors, J. Immunol. 168 (2002) 1940-1949.

[13] T. Pirtilla, T. Numikko, CSF oligoclonal bands, MRI, and the diagnosis of multiple sclerosis, Acta Neurol. Scand. 92 (1995) 468-471.

[14] T. Pouyani, B. Seed, PSGL-1 recognition of P-selectin is controlled by a tyrosine sulfation consensus at the PSGL-1 amino terminus, Cell 83 (1995) 333-343.

[15] T, Revesz, D. Kidd, A.J. Thompson, R.O. Bernard, W.I. McDonald, A comparison of the pathology of primary and secondary multiple sclerosis, Brain 117 (1994) 759-765.

[16] E. Scarpini, D. Galimberti, P. Baron, R. Clerici, M. Ronzoni, G. Conti, G. Scarlato, IP-10 and MCP-1 levels in CSF and serum from multiple sclerosis patients with different clinical subtypes of the disease, J. Neurol. Sci. 195 (2002) 41-46.

[17] J.W. Thorpe, D. Kidd, I.F. Moseley, A.J. Thompson, D.G. MacManus, D.A. Compston, W.I. McDonald, D.H. Miller, Spinal MRI in patients with suspected multiple sclerosis and negative brain MRI, Brain 119 (1996) 709-714.

[18] D.A. Tregouet, S. Barbaux, O. Poirier, S. Blankenberg, C. Bickel, S. Escolano, H.J. Rupprecht, J. Meyer, F. Cambien, L. Tiret, for the Athero*Gene* group, SELPLG gene polymorphisms in relation to plasma SELPLG levels and coronary artery disease, Ann. Hum. Genet. 67 (2003) 504-511.

[19] G.M. Veeldman, K.M. Bean, D.A. Cumming, R.L. Eddy, S.N.J. Sait, T.B. Shows, Genomic organization and chromosomal localization of the gene encoding human P-selectin glycoprotein ligand, J. Biol. Chem. 27 (1995) 16470-16475.