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Multiple sclerosis: Association of gelatinase B/matrix metalloproteinase-9 with risk and clinical course the disease

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ABSTRACT

Background: Multiple sclerosis (MS) is an autoimmune disease characterized by inflammation and axonal degeneration of the central nervous system and a leading cause of disability in young adults. The matrix metalloproteinases in general and specially gelatinase B/metalloproteinase-9 (MMP-9) plays a role in the pathogenesis of multiple sclerosis.
Objective: To investigate the presence of the MMP-9 −1562 C/T polymorphism in a Portuguese population of MS patients and assess its impact in susceptibility and course of the disease. The relation of MMP-9 serum levels with the polymorphism and with clinical and therapeutic factors will also be assessed.
Methods: Our study included 355 Caucasian individuals distributed as MS patients (n=169) and controls (n=186). Samples were genotyped for −1562 C/T polymorphism by PCR-RFLP analysis. MMP-9 concentration in serum was analyzed using a commercially available enzyme-linked immunosorbent assay.
Results: A significant increase in T-allele frequency was found in female MS patients, but not in the total patient population. No association between the presence of the polymorphism and disease progression was found. MMP-9 serum concentrations were increased in patients, and although not influenced by the −1562 C/T polymorphism, were modified by INF-beta therapy.
Conclusion: Although we did not find an association of this polymorphism with disease susceptibility or prognosis, MMP-9 appears to be a good therapeutic response marker for multiple sclerosis.

1. Introduction

Multiple sclerosis (MS) is a progressive autoimmune disease characterized by inflammation, demyelination, and axonal degeneration, resulting in the interruption of myelinated tracts of the central nervous system (CNS) and a leading cause of disability in young adults. The heterogeneous clinical course of the disease and underlying pathophysiological mechanisms, make MS prognosis extremely challenging to define (Hauser and Oksenberg, 2006).

Although the etiology of the disease remains unclear, an autoimmune reaction directed against antigens of cerebral white matter has been proved (Hartung et al., 2004) and the migration of autoreactive immune cells through the blood-brain barrier (BBB) into the CNS seems crucial for the formation of inflammatory lesions (Martin et al., 2000). An important role in this process is played by matrix metalloproteinases (MMPs). In particular, gelatinase B/MMP-9, has been demonstrated to facilitate the influx of inflammatory cells into the CNS (Gijbels et al., 1993; Lee et al., 1999), as well as, the BBB breakdown (Chandler et al., 1995; Gijbels et al., 1993) and to cleave human myelin basic protein *in vitro* (Proost et al., 1993). MMPs are secreted by a wide range of cell types, capable of degrading all protein components of the extracellular matrix (Chandler et al., 1995; de Souza et al., 2005). The

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activity of the MMPs is regulated at different levels (Yong et al., 2001; Yushchenko et al., 2003), such as gene expression, proenzyme activation, and by the activity of the tissue inhibitors of matrix metalloproteinases (TIMPs). The transcriptional activity of the *MMP-9* gene (located in chromosome 20q13) is influenced by two polymorphisms identified in the promoter region. These are a CA_n microsatellite polymorphism from position −90 (St Jean et al., 1995) and a single nucleotide polymorphism at position -1562 caused by a C to T substitution (Zhang et al., 1999). *In vitro* studies have shown that this substitution prevents the binding of a nuclear transcription repressor protein to this region of the *MMP-9* gene promoter, being associated with increased MMP-9 expression (Zhang et al., 1999). On this basis, it is reasonable to hypothesize that both −1562T and the longest repeat alleles may be highly plausible genetic risk factors for MS and therefore, there has been considerable research interest in the possible association of variations in the *MMP-9* gene and MS susceptibility.

A few studies have addressed the involvement of the −1562 C/T polymorphism, alone or in combination with the CAn microsatellite polymorphism, with susceptibility to MS (Benesova et al., 2008; Fiotti et al., 2004; Nelissen et al., 2000; Zivkovic et al., 2007). Despite controversial results (La Russa et al., 2010; Mirowska-Guzel et al., 2009), it is generally accepted that the −1562 C/T polymorphism has no impact on susceptibility to MS (Nischwitz et al., 2015), but its possible influence on disease course is still largely unknown.

At protein level, both gelatinase B/MMP-9 increased concentration (Gijbels et al., 1992; Lee et al., 1999) and activity (Avolio et al., 2003; Liuzzi et al., 2002) in serum and in the cerebrospinal fluid (CSF) of MS patients, compared to controls, have been well established. In fact, this MMP has been suggested as a useful marker for the evaluation of the clinical type, disability and severity of MS (Benesova et al., 2009). However, the relation between MMP-9 levels in peripheral blood and the −1562 C/T and CA_n microsatellite polymorphisms has been addressed only in two small studies (Fernandes et al., 2012; Mirowska-Guzel et al., 2009).

MMP-9 has also been considered a therapeutical response biomarker for interferon-beta (IFN-beta), a common immunomodulatory first-line treatment employed in MS. In fact, serum MMP-9 concentration decrease with IFN-beta therapy (Alexander et al., 2010; Comabella et al., 2009; Yilmaz et al., 2012), correlating with the decrease of active lesions during treatment (Avolio et al., 2005; Trojano et al., 1999).

The aim of our study was to investigate the presence of the −1562 C/T polymorphism in the *MMP-9* gene in a Portuguese population of MS patients, and assess its impact in MS course. We also evaluated serum concentration of MMP-9, and their relation with the −1562 C/T polymorphism, as well as with clinical and therapeutical factors.

2. Materials and methods

2.1. Subjects

This study comprised 355 Caucasian individuals originated from Portugal that represent a genetically stable and homogenous population. MS patients (n=169) were recruited at the Neurology Department of the Centro Hospitalar e Universitário of Coimbra (CHUC)-Coimbra, Portugal and the Braga Hospital-Braga, Portugal. MS was diagnosed according to the McDonald and Polman criteria (McDonald et al., 2001; Polman et al., 2011) and all patients had a minimum clinical follow-up time, of two years. Information regarding patient gender, age at onset, disease duration, initial symptoms (optical *vs* other pathways), subtypes (relapsing-remitting-RR; secondary progressive-SP; primary progressive-PP), severity (estimated using the Expanded Disability Status Scale-EDSS), (Kurtzke, 1983) and treatment, were retrieved from local MS database or individual medical records and included in the analysis. All MS patients had been treated with first-line therapy only (59.7%; including IFN-beta and glatiramer acetate) or with second-line therapy (40.3%; including fingolimod and natalizumab) (Kerbrat et al., 2015). At time of blood collection, 60% of patients were undergoing therapy with IFN-beta, and all RR patients were in remission.

The control group included 186 unrelated healthy volunteers, matched for age, gender and ethnicity. The study protocol was approved by the Hospital (CHUC) Ethics Committee. All participants in this study provided written informed consent.

2.2. Genotyping

Blood samples were collected with EDTA tubes, and genomic DNA was extracted using a standard procedure (Spin Blood Mini Kit, Invisorb®, Stratec molecular - Berlin). The polymorphism at position −1562 C/T (rs3918242 deposited in the NCBI database) in *MMP-9* gene promoter was genotyped by PCR-restriction fragment length polymorphism (RFLP) analysis. PCR amplification, was performed using the primers 5'-ATGGCTCATGCCCGTAATCC-3' (forward) and 5'-GGGCAGGGTCTATATTCACC-3' (reverse), as previously described by (Fernandes et al., 2009; Zhang et al., 1999). The amplified products were digested with *Sph*I (Thermo Scientific, USA), overnight at 37 °C, producing fragments of 224 and 124 bp (T allele) or an undigested fragment of 348 bp (C allele). Fragments were then separated by electrophoresis in 2% agarose gel and visualized through ethidium bromide.

2.3. MMP-9 concentration in serum

MMP-9 concentration in serum (both 92 kDa Pro- and 82 kDa active forms) of 96 MS patients and 63 controls was measured using a commercially available enzyme-linked immunosorbent assay (ELISA), according to the manufacturer's instructions (Quantikine Human MMP-9 Immunoassay, R & D Systems Europe, Ltd. UK).

2.4. Statistical analysis

Statistical analysis was performed using SPSS version 21.0 (IBM). Differences in both allele and genotype frequencies distribution between study groups as well as deviation from Hardy-Weinberg equilibrium were estimated by χ^2 test. Hardy-Weinberg equilibrium was evaluated using a Hardy-Weinberg test Equilibrium Calculator for 2 Alleles (Emerson, 2010). Differences in demographic, clinical variables and MMP-9 serum concentration between groups were compared using the Mann-Whitney *U* test (two groups) or the Kruskal Wallis test (three groups) for continuous variables or the Pearson chi-square (χ^2) test for categorical variables. A two-way ANOVA was used for establishing interactions between diagnosis and MMP-9 genotype. Binary logistic regression models, controlled for age and gender were used to assess the contribution of clinical variables in predicting the −1562 C/T polymorphism carrier status. Survival analysis was used to assess the influence of the −1562 C/T polymorphism in the probability of reaching an EDSS ≥ 3, previously defined as indicative of moderate disability (Kerbrat et al., 2015; Leray et al., 2010). Kaplan-Meier survival curves were plotted and the survival distributions according to the presence or absence of the T allele were compared by the log-rank test. Survival time was calculated as the interval from the initial baseline evaluation to the time to reach an EDSS of 3. For patients who did not reach this score, survival time was censored at the date of the last clinical assessment. The results were presented as mean ± standard error (SEM) and $p \leq 0.05$ was considered statistically significant.

Table 1
Characterization of patients and controls.

	MS	C
	n=169	n=186
Age, years (min-max)	41.44 ± 0.84 (17–69)	39.09 ± 0.96 (20–74)
Gender (Female/male)	121/48	122/64
Subtypes (RR;SP;PP)	143;20;6	–
Age of onset, years (min-max)	32.19 ± 0.78 (8–56)	–
Duration of MS, years (min-max)	11.84 ± 0.64 (2–43)	–
EDSS (min-max)	2.95 ± 0.14 (0–8.0)	–

The data are presented as mean ± standard error.
MS-multiple sclerosis; C-controls; RR-relapsing-remitting.
SP-secondary progressive; PP-primary-progressive.
EDSS-Expanded Disability Status Scale.

3. Results

3.1. Sample characterization and −1562 C/T polymorphism distribution

Demographic and clinical characteristics of MS patients and controls are summarized in Table 1. There were no significant differences in the age and gender distribution between patients and controls.

Genotype distribution of the −1562 C/T polymorphism, was not statistically different between patients (130CC; 35CT; 4TT) and controls (145CC; 37CT; 4TT) and, did not deviate from the Hardy-Weinberg equilibrium in any group. 77% and 78% of patients and controls presented the CC genotype, respectively, and only 2% of patients and controls were homozygous for T allele.

3.2. Genotype, gender and clinical factors

Genotype distribution of the −1562 C/T in patients and controls divided by gender are shown in Table 2. Since the TT genotype frequency is very low (only four cases in both groups), CT and TT carriers were grouped (CT+TT). In the control group, genotype and allelic frequency did not reveal differences between males and females. However, in patients, the frequency of the T allele was 15.7% in females *vs* 5.2% in males (p=0.040) and of the CT+TT genotype was 28.1% in females *vs* 10.4% in males (p=0.014). In fact, none of the male MS patients was homozygous for the T allele, whereas in the control population one out of the four TT cases was a male.

Next, we analyzed the possible influence of the −1562 C/T polymorphism in the disease course. Table 3 shows age of disease onset, duration of MS, EDSS, initial symptoms, subtypes, conversion from RR to SP and therapy, in patients according to T carrier status (CC *vs* CT +TT genotype).

Table 2
Genotype distribution of the −1562 C/T polymorphism in patients and controls by gender.

-1562 C/T *MMP-9*	Genotype	MS n (%)	C n (%)
Total	CC	130 (76.9%)	145 (77.9%)
	CT+TT	39 (23.1%)	41 (22.1%)
Female	CC	87 (71.9%)	98 (80.3%)
	CT+TT	**34 (28.1%)***	24 (19.7%)
Male	CC	43 (89.6%)	47 (73.4%)
	CT+TT	**5 (10.4%)**	17 (26.6%)

MS-multiple sclerosis; C-controls.
* $p \leq 0.05$ female *vs* male in patients.

Table 3
Relation of the −1562 C/T polymorphism with clinical factors in MS patients.

	CC n=130	CT+TT n=39
Age of onset, years (min-max)	32.07 ± 0.88 (8–56)	32.59 ± 1.71 (17–54)
Duration of MS, years (min-max)	12.07 ± 0.73 (2–43)	10.82 ± 1.22 (2–40)
EDSS (min-max)	3.05 ± 0.17 (0–8.0)	2.62 ± 0.25 (0–7.5)
Initial symptoms (optical/other pathways)	(22/78)%	(12/88)%
Subtypes (RR;SP;PP)	107;18;5	35;3;1
Conversion RR-SP (Y/N)	(18/82)%	(8/92)%
Therapy (first-line/second-line)	(50/50)%	(47/53)%

The data are presented as mean ± standard error. RR-relapsing-remitting; SP-secondary progressive; PP-primary-progressive; EDSS-Expanded Disability Status Scale.

Overall, there were no statistically significant differences between groups. To further explore the possible association between the MMP-9 polymorphism and disease course in patients, a logistic regression model was applied, but none of the tested clinical variables (age of disease onset, duration of MS, initial symptoms, subtypes, EDSS and treatment) could independently predict the presence of the −1562 C/T polymorphism. In fact, the only variable that showed an association with this polymorphism was, again, gender (p=0.049). Moreover, Kaplan-Meier survival curves for the probability of reaching an EDSS ≤ 3 were plotted according to the −1562 C/T polymorphism carrier status (not shown). 38% of T non-carriers and 26% of T carriers reached an EDSS ≥ 3 during follow-up, but there was no statistical difference in the time to reach this severity score (CC genotype=184 ± 15 months; CT+TT genotype=346 ± 38 months; χ^2 (1)=1.804; p=0.179).

3.3. Serum gelatinase B/MMP-9 concentration, genotype and clinical factors

Serum gelatinase B/MMP-9 concentration was determined in a subgroup of the study population (63 controls; 96 patients), as shown in Fig. 1. In this subpopulation, MS patients were slightly older than controls (43.92 ± 1.14 years *vs* 39.79 ± 1.77 years; p=0.043), but no differences in gender distribution were seen. Clinical characteristics of

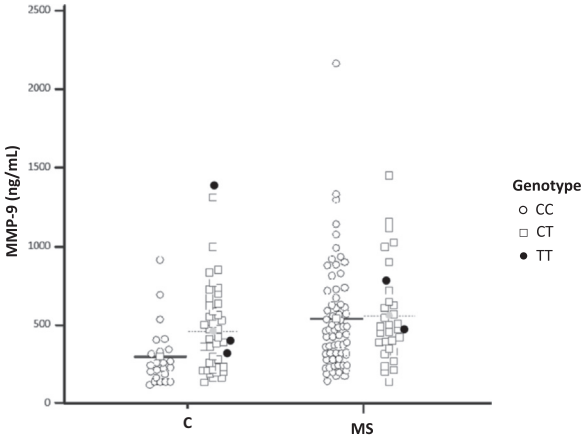

Fig. 1. Serum MMP-9 concentration in a subgroup of the study population (63 controls-C; 96 MS) according to the distribution of genotypes (CC, CT+TT). The horizontal lines indicate the mean concentration of MMP-9 in controls and MS patients.

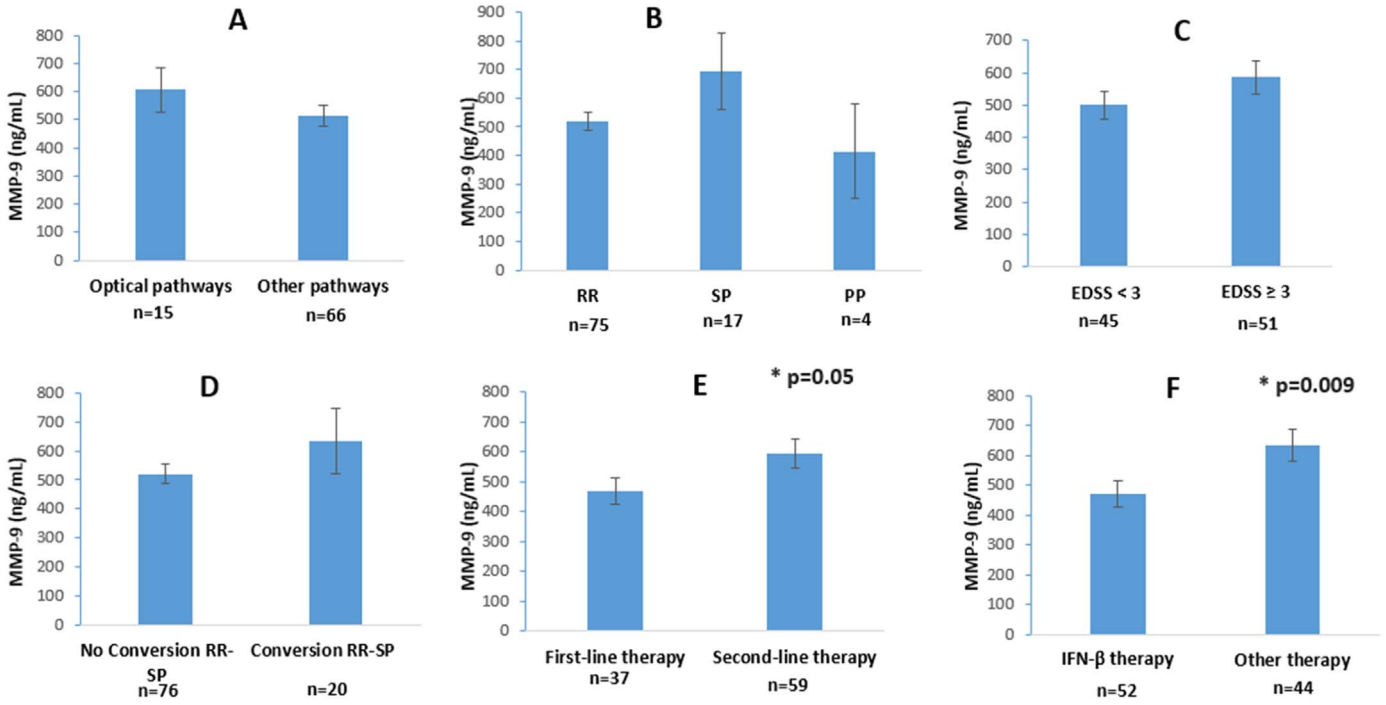

Fig. 2. MMP-9 serum concentration was determined in a subgroup of MS patients (n=96) in relation to initial symptoms (**A**), subtypes (**B**), EDSS < 3; ≥ 3 (**C**), conversion from RR to SP (**D**), therapy (**E**) and IFN-β therapy (**F**). Data are expressed as mean ± SEM. RR-relapsing-remitting; SP-secondary progressive; PP-primary-progressive; EDSS-Expanded Disability Status Scale.

this MS subgroup (not shown) were similar to the total patient population depicted in Table 1.

As expected, mean serum MMP-9 concentration was significantly higher in patients compared to controls (545.19 ± 34.42 ng/mL *vs* 401.10 ± 31.82 ng/mL; p=0.002). Taking into account the −1562 C/T polymorphism, the two-way ANOVA showed that there was a significant effect of diagnosis (p=0.001) and no significant effect of genotype in serum MMP-9 concentration (p=0.079), with no interaction between these two factors (p=0.178). In patients, MMP-9 serum concentration was not influenced by the presence of the T allele, but in the control group, the presence of the T allele was significantly associated with increased concentration of MMP in serum (p=0.008). MMP-9 serum concentration in patients was also not influenced by gender (females=536.92 ± 35.58 ng/mL *vs* males=573.02 ± 92.38 ng/mL; p=0.538).

The relation between MMP-9 serum concentration and disease course is depicted in Fig. 2.

There were no differences in MMP-9 serum concentration associated with MS patient's initial symptoms, subtypes, conversion from RR to SP or disease severity (assessed through reaching an EDSS ≥ 3). There was however a significantly lower MMP-9 concentration in patients currently undergoing IFN-beta therapy (with IFN-beta therapy=470 ± 39 ng/mL *vs* without IFN-beta therapy =634 ± 57 ng/mL; p=0.009). A trend for an association between higher MMP-9 serum concentration with the necessity of second-line therapy was also seen, but failed to reach statistical significance (patients submitted to second-line therapy=594 ± 36 ng/mL *vs* patients submitted only to first-line therapy=466 ± 79 ng/mL; p=0.05). Moreover, MMP-9 serum concentration was not correlated with age of disease onset, duration of MS, or EDSS (not shown).

4. Discussion

In the present study, our results showed that the −1562 C/T polymorphism presented a very similar distribution between patients and controls. However, an increase in the T allele frequency was found in female patients compared to male patients. As expected, MMP-9 serum concentration was increased in patients, and despite not being influenced by the −1562 C/T polymorphism, it was modulated by INF-beta therapy.

The MMP-9 −1562 C/T genotype distribution in our patient population (77% CC; 21% CT; 2% TT) is very similar to what has been described in other MS cohorts from Czech Republic, Brazil or Servia (Benesova et al., 2008; Fernandes et al., 2009; Zivkovic et al., 2007). On the contrary, patients from Italy or Poland were reported to have a much higher T allele frequency (32–45%) (La Russa et al., 2010; Mirowska-Guzel et al., 2009). In our population, the presence of −1562 C/T polymorphism did not reveal susceptibility for MS development, confirming some previous findings (Fernandes et al., 2009; Zivkovic et al., 2007) and in agreement with a recent meta-analysis (Nischwitz et al., 2015). However, our results diverge from other studies finding a decrease (Benesova et al., 2008) or an increase of the T allele in MS patients (La Russa et al., 2010; Mirowska-Guzel et al., 2009).

When we stratified our population by gender, a significant increase in T allele frequency and CT+TT genotype distribution was found in female patients compared to male patients. Besides, comparing the distribution between female patients and controls, we found a slight increase of the T allele in female patients compared to female controls. This result contrasts with other studies, some of which found a significant decrease of T alleles in female patients compared to female controls (Benesova et al., 2008; Nischwitz et al., 2015; Zivkovic et al., 2007), and others reporting no significant differences in allele distributions between female or male patients with MS and healthy volunteers (La Russa et al., 2010; Mirowska-Guzel et al., 2009). Our results need, however, to be interpreted with caution, as the increased risk and incidence of females in MS may interfere with the results. In fact, over two thirds of our MS patients and controls were females, resulting in an overrepresentation of this group in the study population. Moreover, as the T allele frequency is quite low in our population, and the size of our sample is not very large, the association we found between the −1562 C/T polymorphism and the female gender, could be

actually related to a female prevalence in the genotyped groups and not to intrinsic gender characteristics.

The inconsistency of results obtained in different studies could either be due to true genetic variability among different populations or false positive and false negative findings. In particular, population stratification, misclassification and inappropriate statistical methods represent possible causes of false positive results (Abou-Sleiman et al., 2006). Moreover, differences in study design and data analysis could account for the discrepancy between studies. In addition, as MS prevalence decreases from the poles to the equator the presence of MS genetic risk factors might occur at higher frequency in northern europeans (Hauser and Oksenberg, 2006). Therefore, further studies on the regional-variants of putative MS susceptibility genes are still required.

In our work, we did not find any significant association between the −1562 C/T polymorphism and any of the considered clinical variables in MS patients (age of onset, duration of MS, initial symptoms, disease subtypes, severity and treatment). A few other studies have addressed this issue, and also did not find any differences in MS disability status, subtypes, age at onset or disease duration among the genotypes (Benesova et al., 2008; La Russa et al., 2010; Mirowska-Guzel et al., 2009; Zivkovic et al., 2007). On the contrary, in the work of Fernandes, although the genotype was not associated with MS, an association between the TT genotype and a high disability score was observed (Fernandes et al., 2009).

The second part of our study regarding MMP-9 concentration in serum, confirmed that patients have higher concentration than controls, as previously reported by others (Avolio et al., 2003; Benesova et al., 2009; Liuzzi et al., 2002). MMP-9 concentration was not influenced by the −1562 C/T polymorphism neither in the total population nor in patients. However, in controls, MMP-9 concentration was higher in T allele carriers. This association between MMP-9 concentration and −1562 C/T polymorphism has only been previously assessed in two small studies. In the study of Mirowska-Guzel, MMP-9 concentration in peripheral blood was assessed by ELISA in a very small cohort of MS patients (n=15) and a higher concentration was found in T allele carriers (Mirowska-Guzel et al., 2009). Fernandes and collaborators (2012) evaluated plasma MMP-9 activity by zymography and showed higher activity in patients carrying the polymorphism. However, control subjects displayed equal MMP-9 activity among genotypes (Fernandes et al., 2012). A direct comparison with our study is difficult to establish, either due to the small number of patients studied in these previous studies, or to the different methodology and type of samples used (serum *vs*. plasma). In fact, it has been suggested that MMP-9 is released by platelets or leucocytes during serum producing clothing process, thus leading to an overestimation of MMP-9 in serum, that does not correlates to MMP-9 plasma levels in healthy individuals (Gerlach et al., 2007). However, in disease conditions, it was shown that a good correlation exists between serum and plasma concentrations of MMP-9, therefore validating the use of serum sample analysis of MMP-9 in clinical studies (Gerlach et al., 2009).

Our results indicate that, in a physiological situation, the −1562 C/T polymorphism is involved in the regulation of MMP-9 with the C-T substitution being associated with increased MMP-9 expression. However, in patients, this association is lost, and MMP-9 serum concentration becomes independent from this polymorphism. One possible explanation for this fact is that a large proportion of our patients (over 50%) were medicated with IFN-beta, which is, as discussed below, known to target MMP-9. Indeed IFN-beta therapy was associated with a reduction in MMP-9 concentration in our patient population. It is therefore possible that our results have been somehow biased by the influence of the therapeutic agents. If our MS cohort consisted of patients not yet submitted to IFN-beta therapy, the increase in MMP-9 concentration in relation to controls could be even more pronounced and the outcome regarding the influence of the polymorphism could be different.

We did not find any differences in MMP-9 serum concentration in patients in relation to gender, initial symptoms, subtypes or conversion from RR to SP. Furthermore no correlation was seen between MMP-9 serum concentration and age of disease onset or disease duration. Although higher MMP-9 serum concentration was seen in patients undergoing second-line therapy and in patients presenting an EDSS ≥ 3 (patients with EDSS < 3: 500.04 ± 44 ng/mL *vs* patients with EDSS ≥ 3: 585.03 ± 52 ng/mL), they both failed to reach statistical significance. Interestingly, our results have some similarity with the results from Fernandes and collaborators (2012). In their study, when MS participants were analyzed for drug resistance, patients with more than 2 relapses within 2 years, classified as resistant to first-line therapy, presented significantly higher plasma MMP-9 activity, compared to responsive patients. Besides, a correlation was seen between plasma MMP-9 activity and EDSS (Fernandes et al., 2012).

Our study, in line with others, have shown that therapy with IFN-beta induces a decrease in MMP-9 levels (Alexander et al., 2010; Comabella et al., 2009; Galboiz et al., 2001; Yilmaz et al., 2012) as well as an increase in TIMP-1 levels (Alexander et al., 2010; Comabella et al., 2009; Galboiz et al., 2001) contributing to restore BBB integrity. This mechanism might ultimately correlate with the clinical stabilization found in RR patients that respond to IFN-beta therapy (Comabella et al., 2009). Second-line therapy does not act at MMP-9 level (Du Pasquier et al., 2014) and this could explain the increase in MMP-9 concentration in patients submitted to second-line therapy. As second-line therapies are known to be more effective in controlling disease activity and disability progression, a significant correlation between EDSS and MMP-9 concentration might have been concealed.

This study has some important limitations such as the small number of analyzed patients/controls, especially for determination of MMP-9 serum concentration, which could have a strong impact in results consistency and also explain the discrepancies in relation to the existing literature. The study design, including a heterogeneous MS population evaluated by different clinicians and submitted to different therapeutic schemes potentially biased the results and could be improved in the future. Nevertheless, the population was well characterized concerning demographical and clinical variables known to influence disease progression and the possible associations with MMP-9 concentration/polymorphism were thoroughly explored.

5. Conclusion

To our knowledge, this is the first study done in the Portuguese population assessing MMP-9 serum concentration and the MMP-9 −1562 C/T polymorphism in MS patients.

Although we did not find any evidence for an association of MMP-9 −1562 C/T polymorphism with disease susceptibility or prognosis, a significant increase in T allele frequency in female patients was shown. Ultimately, MMP-9 appears to be a good therapeutical response marker for patients with MS treated with interferon beta.

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