# Original Article

# Microsatellite Polymorphism in Haem Oxygenase 1 Gene Promoter in Multiple Sclerosis

(multiple sclerosis / haem oxygenase 1 / *HMOX1* / promoter polymorphism / EDSS / disability progression)

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**Abstract. Previous studies suggested that increased activity of haem oxygenase 1 may ameliorate autoimmune neuroinflammation in experimental models of multiple sclerosis. This increased activity is associated with an augmented number of GT repeats ($\geq 25$) within the *HMOX1* gene promoter. Here we examined 338 patients with multiple sclerosis to determine the influence of their *HMOX1* gene promoter (GT)$_n$ polymorphism and other individual characteristics on the course of the disease. The patients were divided into those with "rapid" or "delayed" course, based on reaching expanded disability status scale step 4 within nine years of disease onset, and the correlations between the disease course and the investigated characteristics were sought using logistic regression analysis. No statistically significant effect of *HMOX1* gene promoter (GT)$_n$ polymorphism on the rate of disability progression was found (P = 0.9). This was confirmed by Cox regression analysis, which did not find any difference in the cumulative risk of reaching expanded disability status scale step 4 between the patients with long and short *HMOX1* gene promoter (P = 0.7). In contrast, covariates significantly associated with the faster disability progression were: progressive course of multiple sclerosis, shorter duration of disease-modifying treatment and older age at disease onset ($P \leq 0.04$). The observed absence of effect of the *HMOX1* promoter (GT)$_n$ polymorphism could be attributed to its known dualistic role in the pathogenesis of autoimmune disorders. As a secondary outcome, we have seen that disease-modifying drugs have the potential to delay disability progression in patients with multiple sclerosis.**

Abbreviations: CNS – central nervous system, DMDs – disease-modifying drugs, EDSS – expanded disability status scale, *HMOX1* – haem oxygenase 1 gene, HO-1 – haem oxygenase 1, MS – multiple sclerosis.

## Introduction

Haem oxygenase 1 (HO-1), also known as heat shock protein 32 (E.C. 1:14:99:3; haem – hydrogen donor: oxygen oxidoreductase), is an inducible, rate-limiting enzyme of haem catabolism transforming haem to biliverdin, free iron Fe$^{II}$ and carbon monoxide CO. Biliverdin is further rapidly metabolized to bilirubin. Products of HO-1 are biologically active agents, which influence tissue redox homeostasis. HO-1 expression within the central nervous system (CNS) is confined to small populations of scattered neurons and glia. This expression is stimulated by various oxidative and noxious stimuli such as endotoxin, hydrogen peroxide, prostaglandins and cytokines (interleukin-1, tumour necrosis factor) (Abraham and Kappas, 2008; Schipper et al., 2009). HO-1 is also known to be elevated in a number of degenerative and non-degenerative neurological disorders, e.g. it has been identified in the glial cells within the multiple sclerosis (MS) plaques (Schipper et al., 2009). The role of HO-1 in demyelinating disorders has been studied and both protective and detrimental effects have been demonstrated. In experimental allergic encephalitis, the rodent model of MS, the inhibition of HO-1 activity suppressed production of free radicals and T-cell influx, increased the level of glutathione and attenuated disease activity (Chakrabarty et al., 2003; Chen et al., 2010). In contrast, Liu and co-workers reported disease exacerbation after similar treatment of Lewis mice with demyelinating disorder (Liu et al., 2001). Another study demonstrated enhancement of de-

*Table 1. Demography of the studied sample*

|  | Overall | Disability progression rate | |
|---|---|---|---|
|  |  | rapid | delayed[†] |
| **Subjects,** number (females:males) | 338 (239:99) | 253 (184:69) | 85 (55:30) |
| **Age,** years ± SD* | 44 ± 10 | 43 ± 11 | 46 ± 10 |
| **Age at MS onset,** years ± SD* | 28 ± 9 | 26 ± 8 | 32 ± 9 |
| **MS type,** number (%)*<br>   relapsing-remitting/secondary progressive<br>   primary progressive | <br>324 (96 %)<br>14 (4 %) | <br>249 (98 %)<br>4 (2 %) | <br>75 (88 %)<br>10 (12 %) |
| **Disease-modifying therapy,** number (%) * | 173 (51 %) | 142 (56 %) | 31 (36 %) |
| **Cytostatics,** number (%)* | 15 (4 %) | 8 (3 %) | 7 (8 %) |

[†] reached EDSS step 4 within nine years of the disease onset
* $P \leq 0.05$, $\chi^2$ tests, *t*-tests

myelination, paralysis and higher mortality after induction of experimental allergic encephalitis in HO-1 gene knockout mice (Chora et al., 2007).

HO-1 is encoded by *HMOX1* located on 22q12 chromosome and consisting of four introns and five exons (Lavrovsky et al., 1996). Three polymorphisms within its promoter area have been described: a $(GT)_n$ dinucleotide length polymorphism and two single nucleotide polymorphisms, T (- 413)A and G (- 1135)A (Exner et al., 2004b). All these polymorphisms have the potential to influence the HO-1 expression (Hirai et al., 2003). The microsatellite polymorphism is based on a variable number of GT repeats; in the majority of populations this may vary from 12 to 40, with the most common alleles with 23 and 30 repeats (Yamada et al., 2000; Chen et al., 2002; Baan et al., 2004; Exner et al., 2004a; Schillinger et al., 2004; D'Silva et al., 2011). The higher $(GT)_n$ dinucleotide repeat length assumes Z-DNA conformation, which is thermodynamically unfavourable compared to B-DNA conformation and attenuates transcriptional activity (Rich et al., 1984; Naylor and Clark, 1990; Delic et al., 1991). As a result, the alleles with more than 25 GT repeats show lower promoter activity in response to the oxidative stress compared to those with less than 25 GT repeats, as was demonstrated *in vitro* (Hirai et al., 2003). In this study, the cells with the higher number of GT repeats were less resistant to the oxidant-induced apoptosis.

The aim of our retrospective study was to examine the influence of the number of $(GT)_n$ repeats within the *HMOX1* gene on the accumulation of disability in patients with MS. We hypothesized that MS patients with the number of $(GT)_n$ dinucleotide repeats exceeding 25 show more rapid progression of disability.

## Material and Methods

### *Subjects*

We have recruited 338 patients with definite diagnosis of MS (239 females and 99 males). The subjects were selected randomly from the patients visiting the MS Centre of the General University Hospital and the First Faculty of Medicine in Prague between 1999 and 2003. All patients signed informed consent and the study was approved by the Ethical Committee of the First Faculty of Medicine, Charles University in Prague and of the General University Hospital in Prague. Only the patients with at least 9-year follow-up or with marked disability [expanded disability status scale (EDSS) step 4 or higher] qualified for the study. Demographic characteristics are shown in Table 1. While 173 patients were receiving disease-modifying drugs (DMDs), 165 patients were not treated with DMDs before they reached EDSS 4 within the nine years of the

*Table 2. Overview of the disease-modifying and immunosuppressive treatment*

| Treatment | No. of patients treated | Average time on treatment per treated patient, years (range) |
|---|---|---|
| IFN-β1a<br>   *Avonex*<br>   *Rebif* | <br>126<br>26 | <br>4.1 (1-8)<br>2.5 (1-5) |
| IFN-β1b | 39 | 3.7 (1-9) |
| glatiramer acetate | 6 | 1.8 (1-5) |
| i.v. immunoglobulins | 9 | 2.0 (1-4) |
| natalizumab | 1 | 1.0 |
| cyclophosphamide | 11 | 2.4 (1-6) |
| mitoxantrone | 3 | 2.3 (1-4) |
| firategrast | 1 | 1.0 |

disease onset. Table 2 gives an overview of the administered DMDs and immunosuppressive drugs.

## Study design

The follow-up of the subjects was done on at least six-monthly basis in our MS centre and included the evaluation of disability with EDSS (Kurtzke, 1983). Time to EDSS step 4 was registered for each patient with EDSS 4 or higher. Patients were stratified into those with "rapid" (EDSS 4 reached within nine years) and "delayed" disability progression (EDSS 4 not reached within nine years); nine years being the median among those who did reach EDSS 4. *HMOX1* gene promoter $(GT)_n$ polymorphism was determined in each patient and its potential influence on the rate of disease progression was evaluated by statistical analysis. To compensate for the variations in age, sex, clinical course of MS (relapsing-remitting or primary progressive course) and treatment, these were included in the analysis as covariates.

## Genetic analysis

Genomic DNA was isolated from peripheral blood leukocytes by standard procedures and genetic analysis was performed as previously described by Král et al. (2011).

## Statistical analysis

The software packages SPSS 17 (SPSS Inc., Chicago, IL) and Statistica 9.1 (StatSoft Inc., Tulsa, OK) were used for all statistical analyses. Effects were considered significant if $P \leq 0.05$. Since only a small number of hypothesis-testing procedures were applied, no multiple testing adjustment was used. Unless indicated otherwise, the results are given as mean ± standard deviation.

Normality of data distributions was assessed in all quantitative variables. To test the effect of the *HMOX1* polymorphism on the rate of disability progression with respect to the relevant covariates (sex, age at MS onset, clinical course of MS, duration of the DMD and immunosuppressant treatment), we used forward stepwise logistic regression analysis. Goodness of model fit was evaluated with Hosmer-Lemeshow $\chi^2$ test. Time to EDSS step 4, adjusted for age, clinical course of MS and DMD treatment, was compared between patients with different *HMOX1* genotypes with Cox proportional hazard model. Comparisons of demographic data between the patients with rapid and delayed disability progression as well as post-hoc comparisons of covariates between the patients with different *HMOX1* genotypes were carried out with *t*-tests and $\chi^2$ tests for the numerical and categorical values, respectively.

## Results

Analysis of the *HMOX1* genotype demonstrated that the most frequent alleles in the studied population were those with 23 and 30 GT repeats (Fig. 1). Eighty-five patients (25 %) reached EDSS step 4 within nine years
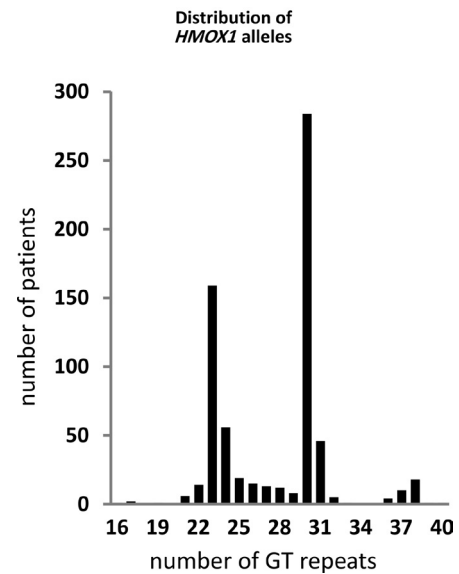


**Distribution of *HMOX1* alleles**

*Fig. 1.* Distribution of *HMOX1* alleles in the studied population. The most prevalent alleles were those with 23 and 30 GT repeats.

of the disease onset. Logistic regression analysis (model fit: $P = 0.8$, $\chi^2_8 = 4.8$, Hosmer-Lemeshow test; predictive value: 77 %) did not show any statistically significant effect of *HMOX1* polymorphism on the rate of disability progression ($P = 0.9$, $Wald_1 = 0.01$). This was confirmed by the analysis of the cumulative risk of reaching EDSS step 4, which did not differ between the patients with different *HMOX1* genotypes (Fig. 2, $P = 0.7$, Wald = 0.14, Cox model). The covariates significantly associated with the rate of disability progression were: clinical course of MS ($b = 1.3$; $P = 0.04$; $Wald_1 = 4.1$), duration of the DMD treatment ($b = -0.2$; $P < 0.001$; $Wald_1 = 13.6$) and age at MS onset ($b = 0.06$; $P < 0.001$; $Wald_1 = 12.9$; logistic regression). It can be seen in Table 1 that among the patients with rapid disability progression there was a higher proportion of those with primary progressive course ($P < 0.0001$, $\chi^2_1 = 16.6$, $\chi^2$ test), a lower proportion of those who received DMDs ($P < 0.001$, $\chi^2_1 = 9.8$, $\chi^2$ test) and a later onset of the first symptoms of MS



**Cumulative risk of reaching EDSS step 4**

*HMOX1* genotype:
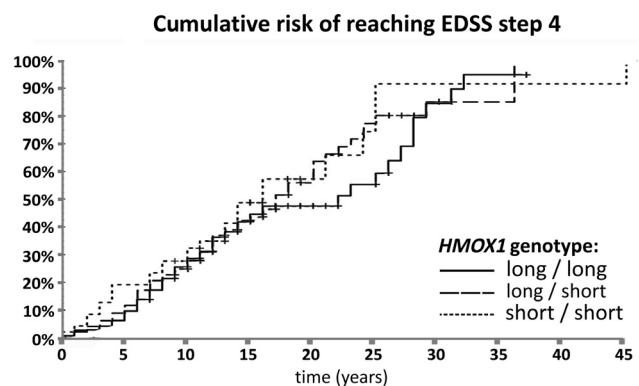— long / long
– – long / short
······· short / short

*Fig. 2.* Cumulative risk of reaching EDSS step 4 compared between patients with different *HMOX1* genotypes. The alleles were categorized as long ($\geq$ 25 GT repeats) and short (< 25 GT repeats). Marks (+) indicate censored subjects.

*Table 3. Comparison of subjects with different HMOX1 promoter polymorphisms*

| | HMOX1 promoter $(GT)_n$ polymorphism | | |
| --- | --- | --- | --- |
| | long/long[†] | long/short[†] | short/short[†] |
| **Subjects,** number (females:males) | 145 (104:41) | 146 (102:44) | 47 (33:14) |
| **Age,** years ± SD | 45 ± 11 | 43 ± 9 | 45 ± 12 |
| **Age at MS onset,** years ± SD | 29 ± 9 | 26 ± 8 | 28 ± 8 |
| **MS type,** number (%) | | | |
| relapsing-remitting/secondary progressive | 138 (95%) | 143 (99%) | 43 (91%) |
| primary progressive | 8 (5%) | 2 (1%) | 4 (9%) |
| **EDSS ≥ 4 before year 9 of MS,** number (%) | 74 (51%) | 69 (47%) | 27 (57%) |

[†] alleles were labelled as long (≥ 25 GT repeats) and short (< 25 GT repeats)

(P < 0.0001, $t_{336}$ = 5.2, *t*-test) compared to those with delayed disability progression. In contrast, sex and duration of immunosuppressive treatment did not have any significant effects on the disability progression rate (P > 0.4; $Wald_1 ≤ 0.6$).

Finally, Table 3 compares the groups of patients with different *HMOX1* genotypes. In agreement with the outcomes of the other analyses, no statistically significant differences were found between the groups. Interestingly, we have observed a trend for the primary progressive MS to be more prevalent in the short allele homozygotes (i.e. < 25 GT repeats) compared to the patients with other genotypes; however, this was not statistically significant (P = 0.6, $\chi^2_2$ = 9.8, $\chi^2$ test).

## Discussion

In this study we did not find any effect of the *HMOX1* $(GT)_n$ polymorphism on the rate of MS progression. The progression was only accelerated by the well-established risk factors, such as late onset of MS or primary progressive MS course. Importantly, treatment with DMDs was associated with lower incidence of rapid disability progression.

This is the first study evaluating the effect of *HMOX1* $(GT)_n$ polymorphism on the rate of progression of MS. To date, several loci associated with increased susceptibility to MS have been identified. Among them the *HLA-DRB1* locus shows the strongest association with disease susceptibility and probably has an impact on the severity of disease course (Wu et al., 2010). Among other susceptibility loci discovered by genome-wide association studies are genes encoding cytokines IL-7, IL-2 and IL-12A, adhesion molecules CD 6 and CD 58 and other molecules such as MPHOSPH9/CDK2AP1 and RGS1 (IMSGC, 2010; Oksenberg and Baranzini, 2010). Studies of copy number variants and network- or pathway-based analyses are expected to reveal more MS susceptibility factors.

Distribution of the *HMOX1* promoter $(GT)_n$ polymorphism in our study was similar to other works, with the most frequent alleles with 23 and 30 repeats (Yamada et al., 2000; Chen et al., 2002; Baan et al., 2004; Exner et al., 2004a; Schillinger et al., 2004). As our population did not show any other predominant number of repeats, unlike some other studied populations (showing 14 or 37 repeats)* (Yamada et al., 2000; Mustafa et al., 2008; D'Silva et al., 2011), we adhered to the conventional dichotomic study design. According to our hypothesis, the alleles with less than 25 $(GT)_n$ repeats, and thus with presumably higher transcriptional activity resulting in increased activity of HO-1, should relate to decreased risk of rapid progression of disability in MS. Concerning immune-mediated disorders, a similar paradigm has previously been applied by Katana et al. (2010), who demonstrated improved function of kidney allografts coming from donors with short *HMOX1* promoters. Furthermore, the protective effects of HO-1 were studied *in vivo* as well as *in vitro* in atherosclerosis, acute coronary syndrome, emphysema, diabetes mellitus and other diseases with inflammatory pathogeneses, and are likely to be mediated by the increased resistance to the oxidative stress (Abraham and Kappas, 2008). However, based on our results, we have rejected this hypothesis in MS. The lack of impact of the *HMOX1* $(GT)_n$ polymorphism on the course of MS could be attributed to the dualistic role of the HO-1 products in autoimmune disorders. In fact, both beneficial and detrimental effects of HO-1 were shown in human MS and in its animal models. In experimental allergic encephalitis, HO-1 and its product carbon monoxide suppress inflammation (Chora et al., 2007) and increase resistance to nitrogen reactive species in oligodendrocytes and motor neurons, thus prolonging their survival (Bishop et al., 2009).

Another product of HO-1, bilirubin, is an intracellular antioxidant and has the ability to inhibit immune effector function by suppressing IL-1 and IL-2 production, decreasing natural killer activity and antibody-dependent cellular toxicity, lymphokine-activated killing activity and DNA synthesis, and therefore is a likely protective factor in inflammatory disease (Maines and Gibbs, 2005; Abraham and Kappas, 2008). The protective effects of bilirubin were observed in allogeneic transplantations both in rats (Shen et al., 2011) and humans (Baan et al., 2004). On the other hand, high bilirubin levels can be neurotoxic, as it was demonstrated in kernicterus – a potentially deadly disease of infants (D'Silva et al., 2011). Finally, iron, which is also produced by HO-1, catalyses the formation of free radicals in oligodendrocytes in experimental allergic encephalitis and its accu-

* This could be attributed to the geographic differences in the distribution of the examined polymorphisms.

mulation in mitochondria leads to neurodegeneration (Levine et al., 2004). Iron deposits are also observed in aging brain and in degenerative disorders such as Alzheimer's and Parkinson's disease (Maines and Gibbs, 2005; Schipper et al., 2009).** It is therefore possible that the iron deposits might take part in the neurodegenerative component of the MS pathogenesis.

Our conclusions are supported by the outcomes of studies done in other autoimmune disorders. For example, in ulcerative colitis, Crohn's disease and other autoimmune disorders of gastrointestinal system, no effects of the *HMOX1* promoter $(GT)_n$ polymorphism on the course and severity of the diseases were observed (Hausmann et al., 2008; Andersen et al., 2010).

As a secondary outcome, we have confirmed the results of other studies, which have shown that DMDs are efficient in reducing progression of disability in patients with multiple sclerosis (Patty and Li, 1993; Jacobs et al., 1996; PRISMS Study Group, 1998). Even though we did not directly compare the time courses of changes in EDSS in patients with and without DMDs, using our dichotomized design we have observed that the proportion of patients with no DMDs was significantly larger among the subjects with rapid than those with delayed accumulation of disability (64 % vs. 44 %), evaluated as EDSS 4 reached within nine years of MS onset. Even if the mechanisms of action of DMDs are not entirely understood, it is known that these drugs suppress pro-inflammatory cytokines, such as TNF-α, IL-1 and others, many of which induce HO-1 expression (Brod et al., 1996; Rep et al., 1996; Chabot et al., 1997). Yet, since the different level of HO-1 expression determined by the $(GT)_n$ polymorphism does not show any measurable impact on the disability progression rate, it is unlikely that this therapeutic effect is mediated through HO-1.

In conclusion, our present study does not support the role of the *HMOX1* promoter $(GT)_n$ polymorphism in the prognosis of patients with multiple sclerosis. A number of potential genetic prognostic markers are likely to emerge from the large multicentric genome-wide studies that are currently underway (IMSGC, 2010; Oksenberg and Baranzini, 2010).

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** On the other hand, also a neuroprotective effect of HO-1 has been described in Parkinson's disease (Quesada et al., 2011).

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