# Arylsulfatase a Gene Polymorphisms in Relapsing Remitting Multiple Sclerosis: Genotype-Phenotype Correlation and Estimation of Disease Progression

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**A B S T R A C T**

*Arylsulfatase A (ASA) is a lysosomal enzyme involved in catabolism of cerebroside-sulfate, major lipid constituent of oligodendrocyte membranes. Various polymorphisms in ASA gene have been described, leading to different levels of enzyme deficiency. Progressive demyelination occurs in metachromatic leukodystrophy (MLD), while the condition of ASA-pseudodeficiency (ASA-PD) is suggested to contribute to complex pathogenesis of multiple sclerosis (MS). This work presents usefulness of genotype-phenotype correlation in estimation of disease severity and progression. The presence of two most common mutations associated with ASA-PD was analyzed in 56 patients with diagnosis of relapsing-remitting multiple sclerosis, by polymerase chain reaction restriction fragment length polymorphism method. In MS patients confirmed as ASA-PD mutations carriers, arylsulfatase activity was determined in leukocyte homogenates by spectrophotometry. To determine whether there is a difference between disability level and/or disease progression in patients with or without mutations we have estimated disability level using Expanded disability status scale (EDSS) and disease progression using Multiple sclerosis severity score (MSSS). Correlation of genotypes and disease progression was statistically analyzed by Kruskal-Wallis test. Patients showing higher MSSS score and found to be carriers of both analyzed ASA-PD mutations were additionally examined using conventional magnetic resonance (MR) techniques. The presence of either one or both mutations was determined in 13 patients. Lower ASA activities were observed in all MS patients carrying the mutations. Nine of the mutations carriers had mild disability (EDSS=0–4.0), 1 had moderate disability (EDSS=4.5–5.5), and 3 had severe disability (EDSS≥6.0). On the other hand, only 3 MS patients who were mutation carriers showed MSSS values lower than 5.000 while in other MS patients-mutation carriers the MSSS values ranged from 5.267 to 9.453. Comparison of MR findings between MS patients, mutations carrier vs. non-carrier, matched for sex, age and disease duration, showed that the total number of lesions and the number of hypointense lesions on T1-weighted images was greater in MS patient carrying the ASA-PD mutations. Our results on genotype-phenotype correlation analysis indicate a possible contribution of detected arylsulfatase A gene polymorphisms to the clinical severity of multiple sclerosis, estimated by EDSS, MSSS and MR findings. The MSSS proved to be more appropriate indicator of disease progression and should be more frequently used in clinical practice especially for comparison of disease progression in different groups of patients and identification of factors that may influence disease progression such as the presence of gene polymorphisms.*

*Key words: multiple sclerosis, arylsulfatase A, gene polymorphisms, genotype-phenotype correlation, disease progression, multiple sclerosis severity score, magnetic resonance techniques*

## Introduction

Arylsulfatase A (ASA, EC 3.1.6.1) is a lysosomal enzyme involved in catabolism of cerebroside-sulfate[1]. This major lipid constituent of oligodendrocyte membranes contributes to maintenance of myelin sheath integrity[1,2]. Various polymorphisms in ASA gene have been described, leading to different levels of enzyme deficiency[3,4]. Progressive demyelination occurs in metachromatic leukodystrophy (MLD) due to lysosomal storage of sulfatide which mainly accumulates in oligodendrocytes and Schwann cells[1,5,6]. Interestingly, low ASA activities have also been reported in healthy individuals without clinical signs of MLD, due to condition termed ASA pseudodeficiency (ASA-PD)[1,7]. Two mutations in the ASA gene, responsible for the majority of ASA-PD alleles, are designated as N350S and 1524+95 A-G mutation[7]. Frequency of these two mutations is reported to be relatively high in different populations of Caucasian origin (7.3–15 %)[7]; in the Croatian population it has been previously estimated at 6.4 % for N350S and 2.8 % for 1524+95 A-G mutation[8].

The ASA-PD condition is suggested to contribute to complex pathogenesis of multiple sclerosis (MS), a chronic neurological disorder characterized by demyelination in central nervous system[9]. It has been hypothesized that different mutations in ASA gene can influence cell vulnerability and thus facilitate main pathologic process/mechanism occurring in multiple sclerosis. By suggested mechanism, mutations associated with ASA-PD may cause death of small populations of oligodendrocytes and release myelin antigens which may activate immune system. Genotype-phenotype correlation is thus useful in estimation of disease severity and progression, and can be more accurately analyzed using recently introduced Multiple sclerosis severity score method (MSSS)[10]. The MSSS method has been developed since there is no consensus method for determining progression of disability in MS patients when each patient has had only a single assessment of disability estimated by Kurtzke expanded disability status scale (EDSS)[11] in the course of the disease. This MSSS method relates scores on the EDSS to the distribution of disability in patients with comparable disease durations. It is a powerful method for detecting different rates of disease progression in different groups of patients.

The aim of this work was to analyze the impact of detected polymorphisms in arylsulfatase A gene to the clinical severity of multiple sclerosis, estimated by EDSS, MSSS and MR findings. The work supports the utilization of genotype-phenotype correlation analysis which provides useful data for estimation of disease progression and identification of factors influencing disease progression.

## Patients and Methods

We have analyzed the presence of two most common mutations associated with ASA-PD (N350S and 1524+95 A-G) in 56 patients with diagnosis of relapsing-remitting multiple sclerosis (RRMS). The patients were recruited at the University Department of Neurology, »Sveti Duh« General Hospital, Zagreb. The patient group comprised of 40 females (age range: 22–57 years) and 16 males (age range: 18–68 years). The clinical diagnosis of multiple sclerosis was given in accordance with McDonald's criteria[12,13]. Blood samples were collected by venipuncture during morning hours and before any medications were given. The study was approved by the Hospital ethical committee and informed consent was taken from each patient.

### Determination of mutations associated with arylsulfatase A pseudodeficiency

The presence of ASA-PD mutations was determined by polymerase chain reaction restriction fragment length polymorphism method (PCR-RFLP), as previously described[7]. Genomic DNA was extracted from whole blood samples using modified standard salting out procedure[14]. After PCR amplification, RFLP was performed using *BsrSI* restriction enzyme for the N350S mutation and *DdeI* restriction enzyme for the 1524+95 A-G mutation (Figure 1). In the presence of N350S mutation, a *BsrSI* restriction site is created and a 275 bp amplified fragment yields 161 bp and 114 bp fragments. In the case of 1524+95 A-G mutation, a *DdeI* restriction site is generated, and 114 bp amplified fragment is cleaved to two smaller fragments of 97 bp and 17 bp.

### Determination of arylsulfatase A activity in leukocyte homogenates

Leukocytes were isolated from blood samples of the patients found to be ASA-PD mutations carriers. Leukocyte pellets were dispersed in 0.25% Triton X-100 and ho-
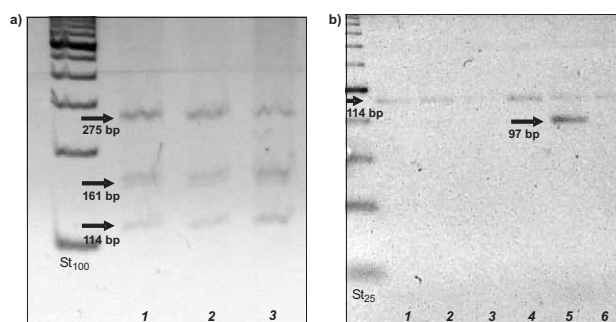


*Fig. 1. Detection of mutations associated with arylsulfatase A pseudodeficiency: a) N350S and b) 1524+95 A-G. The results of PCR-RFLP analysis are shown for several samples derived from patients with diagnosis of multiple sclerosis. Specific restriction products were separated by agarose gel-electrophoresis and visualized by ethidium bromide. Left panel: lanes 1–3 show heterozygous N350S genotype (275 bp amplified fragment yields 161 bp and 114 bp fragments in the presence of mutation). Right panel: lanes 1–4 and 6 are homozygous for non-mutated genotype and lane 5 is a heterozygous 1524+95 A-G genotype (in the presence of mutation, 114 bp amplified fragment is cleaved to two smaller fragments of 97 bp and 17 bp, the latter not visible on gel because of its small size). $St_{25}$ and $St_{100}$ – DNA standards (25 bp and 100 bp), bp – base pairs.*

mogenized. Proteins were determined in leukocyte homogenates according to the method of Lowry[15], using bovine serum albumin (BSA, 1 mgmL$^{-1}$) as a standard. The activity of arylsulfatase A was measured by spectrophotometry ($\lambda$=515 nm) using *p*-nitrocatechol sulfate as chromogenic substrate[16]. The results on arylsulfatase A activity were expressed as nanomoles of degraded substrate *per* hour *per* milligram protein (nmol h$^{-1}$ mg$^{-1}$).

### Estimation of disability level and disease progression in patients with multiple sclerosis

To determine whether there is a difference between disability level and/or disease progression in patients with or without mutations we have estimated disability level using Expanded disability status scale[11] as well as disease progression using Multiple sclerosis severity score[10] for all patients. Correlation of genotypes and disease progression was statistically analyzed by Kruskal-Wallis test (MSSS test, computer programme, version 3.0)[17].

### Magnetic resonance imaging techniques

A preliminary qualitative comparison of radiological findings was made using MR exams of one patient with mutation and of an age, sex and disease duration corresponding control patient. Both exams were done using conventional MR techniques (T1-, T2-weighted and FLAIR sequences in multiple planes, with gadolinium enhancement). Imaging markers of disease severity were graded, namely atrophy, number of hyperintense lesions on T2--weighted images, number of hypointense lesions on T1-weighted images, and number of gadolinium enhancing lesions.

## Results

The presence of either one or both analyzed ASA-PD mutations was determined in 13 out of 56 MS patients (Figure 1). All 13 MS patients with mutations were found to be heterozygous carriers of either/both mutations. Demographic characteristics of 13 MS patients (gender, age, disease duration) found to be ASA-PD mutation carriers are given in Table 1. Both ASA-PD mutations were detected in 8 MS patients, only N350S mutation was found in 4 patients, while 1 patient carried only 1524+95 A-G mutation (Table 2).

The correlation between genotype and enzyme activity was analyzed in the group of MS patients-mutations

**TABLE 1**
DEMOGRAPHIC CHARACTERISTICS OF MULTIPLE SCLEROSIS PATIENTS – CARRIERS OF ARYLSULFATASE A MUTATIONS

| Gender | Male (N=3) | Female (N=10) |
|---|---|---|
| Age range (years) | 34–47 | 26–57 |
| Mean age (years) | 52.3 | 43.3 |
| Duration of the disease (range in years) | 8–15 | 1–12 |
| Duration of the disease (mean in years) | 11 | 5.8 |

**TABLE 2**
CORRELATION OF DETECTED MUTATIONS AND ARYLSULFATASE A ACTIVITY IN MULTIPLE SCLEROSIS PATIENTS

| Detected mutation in arylsulfatase A gene | MS patients-mutation carriers (N=13) | Arylsulfatase A activity (nmol h$^{-1}$ mg$^{-1}$) median (range) |
|---|---|---|
| N350S and 1524+95 A-G | 8 | 83 (57–137) |
| N350S | 4 | 93 (82–121) |
| 1524+95 A-G | 1 | 62 |

MS – multiple sclerosis

carriers (Table 2). Determined ASA activities in 13 MS patients-mutations carriers ranged from 57–137 nmol h$^{-1}$ mg$^{-1}$ (median value: 83.0 nmol h$^{-1}$ mg$^{-1}$) and were found to be in the lower part of the ASA activity reference range (60–300 nmol h$^{-1}$ mg$^{-1}$)[1,4]. In 4 MS patients, heterozygous carriers of only the N350S mutation, ASA activities were in the range of 82–121 nmol h$^{-1}$ mg$^{-1}$ (median value: 93.0 nmol h$^{-1}$ mg$^{-1}$); in 8 MS patients, carriers of both mutations, the range of ASA activities was 57–137 nmol h$^{-1}$ mg$^{-1}$ (median value: 83.5 nmol h$^{-1}$ mg$^{-1}$); ASA activity was also low in 1 patient-heterozygous carrier of only 1524+95 A-G mutation (62 nmol h$^{-1}$ mg$^{-1}$).

Estimation of disability level using Expanded disability status scale showed that 9 of the mutations carriers had mild disability (EDSS=0–4.0), 1 had moderate disability (EDSS=4.5–5.5), and 3 had severe disability (EDSS$\geq$ 6.0) (Table 3). Although we found no statistically significant correlation of genotypes and MSSS in the group of 56 MS patients (Kruskal-Wallis test), we observed that only 3 MS patients who were mutation carriers showed MSSS values lower than 5.000 while in other MS patients-mutation carriers the MSSS value ranged from 5.267 to 9.453 (Table 3).

Patients found to be carriers of both analyzed ASA--PD mutations and showing higher MSSS score were additionally examined using conventional magnetic resonance techniques. Comparison of MR findings between
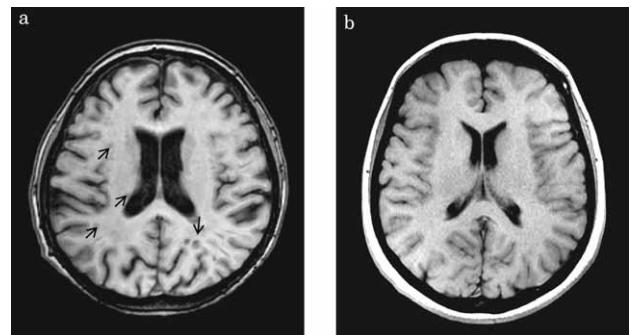


*Fig. 2. Axial T1-weighted MR images in multiple sclerosis patients confirmed as a) arylsulfatase A mutations carrier and b) arylsulfatase A mutations non-carrier, matched for age, gender and disease duration. Note white matter »black holes« (marked by arrows) in a) patient with mutations.*

**TABLE 3**
ESTIMATION OF DISABILITY LEVEL AND DISEASE
PROGRESSION IN MULTIPLE SCLEROSIS PATIENTS-CARRIERS
OF ARYLSULFATASE A MUTATIONS

| Mutation N350S | Mutation 1524+95 A-G | EDSS | MSSS |
|---|---|---|---|
| | | Mild disability (0–4.0) | |
| M | NM | 2.0 | 5.866 |
| M | M | 2.0 | 5.866 |
| M | M | 3.0 | 4.568 |
| NM | M | 3.0 | 4.966 |
| M | NM | 3.0 | 5.382 |
| M | M | 4.0 | 4.621 |
| M | M | 4.0 | 5.267 |
| M | NM | 4.0 | 7.652 |
| M | M | 4.0 | 8.138 |
| | | Moderate disability (4.5–5.5) | |
| M | M | 5.0 | 6.501 |
| | | Severe disability (≥6.0) | |
| M | NM | 6.0 | 7.652 |
| M | M | 7.0 | 8.169 |
| M | M | 7.0 | 9.453 |

M – mutation, NM – no mutation, EDSS – Expanded disability status scale, MSSS – Multiple sclerosis severity score

MS patients, mutations carrier *vs.* non-carrier, matched for sex, age and disease duration, showed no significant volume loss of brain tissue in both patients. However, the patient with mutation had greater total number of lesions in all 3 assessed categories (hyperintense lesions on T2-weighted images, hypointense lesions on T1-weighted images, enhancing lesions) and greater number of lesions in every separate category. Most notable difference was in number of hypointense lesions on T1-weighted images as the patient with mutation had many such lesions and control patient none (Figure 2).

## Discussion and Conclusion

This work indicates the advantages of genotype-phenotype correlation analysis supported by MSSS estimation and MR findings in patients with relapsing-remitting multiple sclerosis. We analyzed the influence of arylsulfatase A gene polymorphisms to the severity and progression of disease in a group of MS patients. We found that MS patients who were carriers of mutations associated with arylsulfatase A pseudodeficiency also exhibited lower enzyme activities. Genotype-phenotype correlation analysis was completed by estimation of disability level and disease progression using EDSS and MSSS, and preliminary qualitative analysis of magnetic resonance findings.

Possible contribution of arylsulfatase A gene polymorphisms to the complex pathogenesis of neurodegenerative disorders has been previously discussed[8,18–20]. Differ-

ent levels of enzyme deficiency, due to mutations in ASA gene, could lead to long-term accumulation of non-degraded substrate and thus influence the cellular vulnerability. In multiple sclerosis, the same mechanism could underlie a death of oligodendrocyte subpopulations thus enabling liberation of myelin antigens and stimulation of immune response[9]. In this study, the correlation between ASA-PD genotype and phenotype was indeed confirmed, as lower enzyme activities were observed in ASA-PD mutation carriers.

In order to investigate influence of analyzed ASA-PD gene polymorphisms to the severity and progression of disease, we estimated disability level in MS patients by EDSS and MSSS. Although EDSS is useful in determining clinical signs of disability level in multiple sclerosis, it shows limitations related to single assessments in the course of the disease which give no insight into disease progression. Moreover, EDSS may vary significantly depending whether estimated during relapse or remission of the disease. Recently introduced Multiple sclerosis severity score method has been developed for the purposes of more accurate estimation of disease progression[10]. The MSSS relates scores on the EDSS to the distribution of disability in patients with comparable disease durations. Since MSSS method takes into account disability as well as the duration of the disease it is a more appropriate indicator of disease progression than EDSS which presents only the disability level. The MSSS is also a powerful method used for correlation analysis of groups of patients with different genotypes and disease progression and is proven to be useful for identifying factors that may influence disease progression such as the presence of gene polymorphisms. In our study, MSSS was helpful in estimation of disease severity as it confirmed that MS patients-mutations carriers indeed showed higher MSSS values indicating faster disease progression.

Additionally, the analysis of brain magnetic resonance imaging data is important in clarifying the correlation of morphological changes (demyelination) with the presence of gene polymorphisms. Our preliminary qualitative analysis of radiological findings showed that patient-mutations carrier compared with patient-mutation non-carrier had greater total number of demyelinating lesions, with most notable difference in number of hypointense lesions on T1-weighted images. Lesions on T1-weighted images are seen as areas of low signal intensity compared to normal-appearing white matter. These so-called »black holes« have various pathological substrates depending on their age. In acute stage this hypointensity represents edema with or without myelin destruction and axonal loss. In most cases, these acute lesions become isointense and disappear with resolution of inflammatory process, while only smaller proportion develop into persisting or chronic lesions, which correlate pathologically with areas of irreversible tissue damage. It is known that chronic »black holes« are more frequent in patients with progressive disease than in those with relapsing-remitting disease and it has been suggested that their number could serve as a surrogate marker of disability progression[21,22].

Here presented radiological analysis in which MS patient/mutation carrier was compared with MS patient/mutation non-carrier, carefully matched for age, gender, disease duration and MSSS value, suggests the correlation of present ASA-PD mutations, low ASA activity, high MSSS value and morphological finding of irreversible axonal degeneration. Further morphological investigation should include larger group of MS patients, and utilize the advantage of more powerful MR device and three-dimensional MR volumetric analysis of lesions.

In conclusion, our results indicate a possible contribution of detected arylsulfatase A gene polymorphisms to the clinical severity of multiple sclerosis. The MSSS proved to be more appropriate indicator of disease progression and should be more frequently used in clinical practice especially for comparison of disease progression in different groups of patients and identification of factors that may influence disease progression such as the presence of gene polymorphisms. Presented approach of genotype-phenotype analysis enabled more accurate estimation of disease severity and progression in here investigated group of MS patients; also it served for comparison of disease progression in different groups of patients and identification of factors that may influence disease progression.

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**POLIMORFIZMI U GENU ZA ARILSULFATAZU A I RELAPSNO-REMITIRAJUĆA MULTIPLA SKLEROZA: KORELACIJA GENOTIPA I FENOTIPA U SVRHU PROCJENE PROGRESIJE BOLESTI**

**S A Ž E T A K**

Arilsulfataza A (ASA) je lizosomski enzim uključen u razgradnju cerebrozid-sulfata, glavnog lipidnog sastojka oligodendrocitnih membrana. Opisani su brojni polimorfizmi u genu za arilsulfatazu A koji imaju za posljedicu različite razine nedostatne aktivnosti enzima. Tako se kod metakromatske leukodistrofije (MLD) razvija progresivna demijelinizacija, a za ASA-pseudodeficijenciju (ASA-PD) pretpostavlja se da je povezana sa složenom patogenezom multiple skleroze (MS). Ovaj rad ukazuje na korisnost analize korelacije genotipa i fenotipa u procjeni težine i progresije bolesti.

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Prisutnost dviju najčešćih mutacija povezanih s ASA-PD ispitivana je metodom analize duljine restrikcijskih fragmenata u 56 pacijenata s dijagnozom relapsno-remitirajućeg oblika multiple skleroze. U pacijenata za koje je utvrđeno da su nositelji ASA-PD mutacija, određena je aktivnost arilsulfataze A u leukocitnim homogenatima spektrofotometrijskom metodom. Utvrđivanje razlika stupnja invalidnosti i/ili progresije bolesti u pacijenata sa ili bez ASA-PD mutacija temeljeno je na procjeni stupnja invalidnosti primjenom EDSS (Expanded disability status scale) i MSSS (Multiple sclerosis severity score) zbroja. Korelacija genotipa i progresije bolesti analizirana je statistički Kruskal-Wallis-ovim testom. Pacijenti s višim MSSS vrijednostima za koje je utvrđeno da ujedno imaju obje ASA-PD mutacije dodatno su pregledani konvencionalnim tehnikama magnetske rezonancije (MR). Prisutnost jedne ili obje ASA-PD mutacije utvrđena je u 13 pacijenata. U ovih je pacijenata nađena i niža aktivnost arilsulfataze A. Također je u 9 pacijenata zabilježena blaga invalidnost (EDSS=0–4,0), 1 je pacijent imao umjereni stupanj invalidnosti (EDSS=4,5–5,5), a 3 pacijenata tešku invalidnost (EDSS≥6,0). Samo 3 pacijenta nositelja mutacija imali su MSSS vrijednost nižu od 5,000, dok su MSSS vrijednosti ostalih nositelja mutacija bile u rasponu od 5,267 do 9,453. Usporedba nalaza MR-e između pacijenta nositelja mutacija i pacijenta bez mutacija, usklađenih po spolu, dobi i duljini trajanja bolesti, pokazala je da je ukupan broj lezija kao i broj hipointenzivnih lezija na T1-snimkama veći u pacijenta koji je nositelj ASA-PD mutacija. Rezultati analize korelacije genotipa i fenotipa ukazuju da utvrđeni polimorfizmi u genu za arilsulfatazu A mogu utjecati na težinu kliničke slike multiple skleroze, procjenjenu pomoću EDSS, MSSS i MR nalaza. MSSS zbroj dokazan je kao bolji pokazatelj progresije bolesti i trebao bi se češće koristiti u kliničkoj praksi naročito za usporedbu progresije bolesti među različitim skupinama bolesnika te za prepoznavanje faktora koji mogu utjecati na progresiju bolesti poput prisutnosti genskih polimorfizama.