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White Paper No2

Circulatory indicators of lipid peroxidation and fatty acid profile between patients with relapse remitting and progressive multiple sclerosis

Genetic analysis of functionally relevant variants in ferroptosis-related genes and variants associated with MS disease severity

Second public survey “Science for Citizens: Genes and Multiple Sclerosis” analytics included

This research is supported by the Science Fund of the Republic of Serbia, #GRANT No. 7753406, Identification and functional characterization of extracellular and intracellular genetic regulators of ferroptosis related processes in multiple sclerosis - FerroReg

https://ferroreg.vin.bg.ac.rs/

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About Project

The overall objective of the FerroReg project is to improve knowledge on genetic architecture and intracellular and extracellular regulation of ferroptosis related molecular processes and pathways, which promote detrimental effects on oxidative defense, mitochondrial function, fatty acid metabolism and lipid peroxidation which could lead to progression and disability in multiple sclerosis (MS). Ferroptosis, as an iron-dependent type of molecularly controlled but not developmentally programmed cell death, discovered in 2012, was recently recognized as driver for neurodegeneration, a hallmark of this chronic inflammatory disease. Genetic regulators of ferroptosis as orchestrated process have not been investigated yet, either in cell cultures or in humans.

Mission

Nowadays, the genetic background as possible risk factor becomes inevitable part in prevention strategies. The molecular components of the ferroptosis are those that have been separately shown to be targets for supplementation modulation, nutritional and lifestyle influence, which are equally important therapeutic avenue for human diseases. Currently there is no cure for MS. All available treatments are disease modifying and they mainly target inflammation while neurodegeneration is not controlled. So, there is an urgent need for novel medicine and supplementation associated strategies to treat the symptoms and reduce the number of relapses (disease-modifying therapies), which are associated with neuronal loss.

In this white paper you will find:

1. **A**) Analysis of circulatory molecular indicators of the main ferroptosis-related processes, as contributors to the clinical manifestation of MS and differences between RRMS and PMS disease course, including

   lipid peroxidation:
   - malondialdehyde (MDA),
   - 4-hydroxynonenal (4-HNE), and
   - hexanoyl–lysine adduct (HEL)),

   glutathione-related antioxidant defense:
   - total glutathione (reduced (GSH) and oxidized (GSSG) and
   - glutathione peroxidase 4 (GPX4)

   iron metabolism:
   - iron,
   - transferrin and
   - ferritin

   B) The erythrocyte fatty acid (FA) profile in multiple sclerosis linked to the disease course (RRMS and SPMS), lipid peroxidation, and dietary influence

2. Association of functionally relevant gene variants in ferroptosis-related genes along with DYSF–ZNF638 and MTSS1 with severity of multiple sclerosis and target gene mRNA expression
3. Analytics of a second short public survey "Science for Citizens: Genes and Multiple Sclerosis" which address basic knowledge about MS, genetics, therapy options and research related to MS in Serbia.

Methodology

The methodology underpinning this white paper is rooted in comprehensive research, high end molecular biology technics, cross-sector collaboration, and rigorous analysis. FerroReg project has a multi-phased approach.

- **Scientific Research:** comprehensive elucidation of unknown ferroptosis regulatory patterns by experimentally gained results, research of existing databases, academic literature and by multidisciplinary approach.
- **Analytical Framework:** Using a blend of qualitative and quantitative methods, statistics and bioinformatics we construct knowledge and robust models to dissolve the complex problems improving the diagnosis, prevention and treatment of multiple sclerosis and accelerating the development of therapeutics and supplementation nutritional and lifestyle modulation.
- **Beneficiaries and Stakeholder Engagement:** Recognizing the importance of varied perspectives and effective cooperation between science and society our team engages with a broad range of citizens, communities, public stakeholders and private stakeholders to obtain insights into what affects health at the local level, to contribute to community development and decisions of policymakers.
- **Solution Formulation:** Relaying on our experimental results, we will propose novel predictive molecular disease severity indicators to improve preventive and disease modifying strategies toward precision supplementation and nutrigenomics.

Results

1. **Circulatory indicators of lipid Peroxidation, and erythrocyte profile of fatty acids in patients with relapsing-remitting (RRMS) and progressive (PMS) multiple sclerosis**

A) Ferroptosis, a lipid peroxidation- and iron-mediated type of regulated cell death, relates to both neuroinflammation, which is common in relapsing-remitting multiple sclerosis (RRMS), and neurodegeneration, which is prevalent in progressive (P)MS. We have analyzed circulatory molecular indicators of all main processes associated with ferroptosis: lipid peroxidation (malondialdehyde (MDA), 4-hydroxynonenal (4-HNE), and hexanoyl–lysine adduct (HEL)), glutathione-related antioxidant defense (total glutathione (reduced (GSH) and oxidized (GSSG)) and glutathione peroxidase 4 (GPX4)), and iron metabolism (iron, transferrin and ferritin) to estimate their contributions to the clinical manifestation of MS and differences between RRMS and PMS disease course. Disease-modifying therapies and detailed demographic and

clinical data were taken into account to estimate interactions with the target molecular parameters in terms of MS course and severity.

B) Fatty acids and their metabolites are the established contributors of the central nervous system (CNS) chronic inflammation, degeneration, and demyelination, which represent the hallmark pathogenic processes in multiple sclerosis. FerroReg has investigated the possible changes in the profile of long-chain fatty acids in the erythrocytes, according to the clinical course of disease - RRMS and PMS, and to test whether the fatty acid levels correlate with the levels of lipid peroxidation indicator, 4-HNE, and the clinical parameters. We considered erythrocytes as a preferable source, as these cells are able to provide the levels of fatty acids that are more stable with respect to dietary changes, hence varying more slowly than those in serum/plasma, and reflecting the average levels or metabolism of fatty acids over a longer time period.

- We found significantly decreased plasma levels of 4-HNE, a known product of lipid peroxidation, in patients with progressive MS compared to RRMS (**Figure 1**.) and a negative correlation of 4-HNE with EDSS (Stojkovic L, 2024). The detected decrease in circulating 4-HNE would be an increase in cellular could be consequence of 4-HNE-protein adduct formation due to excessive oxidative stress established in the progressive course of MS, along with assumed less efficient 4-HNE metabolism. In addition, we have also found a reduction of 18:2n-6 linoleic acid (LA) (polyunsaturated, omega−6 fatty acid) in progressive MS patients, which represents one of the main sources of the reactive carbonyl species, 4-HNE (Stojkovic et al., 2025, under review). Hence, decrease in 18:2n-6 (LA) could be linked with a decrease in circulating 4-HNE, observed in the same group of PMS patients. Namely, the presumed increased CNS uptake of n-6 such as 18:2n-6 (LA), in PMS patients could, under conditions of enhanced oxidative processes, lead to an increase in 4-HNE and its consequent negative outcomes in the CNS cells, including the oxidative stress and neurodegeneration, which are expected in the progressive course of MS.

- In accordance with this possible explanation are the obtained correlations: the erythrocyte total n-6 fatty acids and 22:4n-6 adrenic acid (ADA) correlated negatively with both EDSS and MSSS in the PMS group, and the correlations with MSSS remained significant after adjustment for age and BMI, thus linking lower levels of circulating n-6 with a more severe clinical phenotype of the PMS course (Stojkovic et al., 2025, under review).

- Total glutathione and oxidized gluthatione (GSSG) was also lower in PMS compared to RRMS patients, while GSH and GPX4 levels remained unchanged. GSH was inversely correlated with EDSS in RRMS patients; hence, based on the current findings, we propose that treatment with agents acting to increase GSH, such as fumarate, might be beneficial, particularly for RRMS patients.

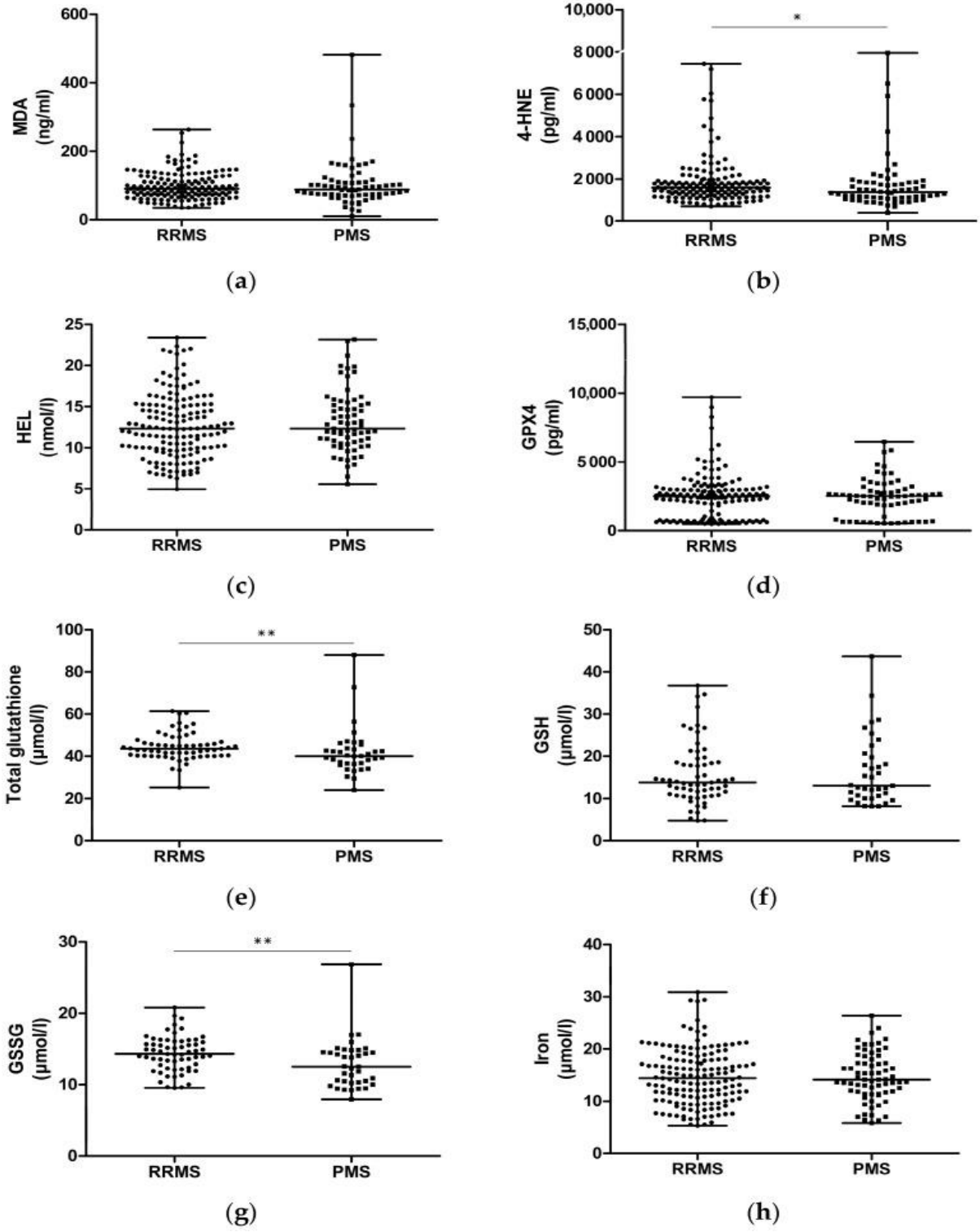

Figure 1. Molecular parameters in patients with RRMS and PMS: (a) Malondialdehyde (MDA); (b) 4-Hydroxynonenal (4-HNE); (c) Hexanoyl-lys adduct (HEL); (d) Glutathione peroxidase 4 (GPX4); (e) Total glutathione, GSH + GSSG; (f) Reduced glutathione (GSH); (g) Oxidized glutathione (GSSG); (h) Iron; (i) Transferrin; (j) Ferritin. RRMS—relapsing–remitting multiple sclerosis; PMS—progressive multiple sclerosis; values of parameters are presented with median and range (minimum–maximum); p-values (Mann–Whitney U test) < 0.05 are considered statistically significant: * $p < 0.05$, ** $p < 0.01$.

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- We haven`t found significant changes in serum levels of iron, transferrin, and ferritin between the two analyzed groups, RRMS and PMS patients.
- Compared with RRMS patients, the PMS group had significant increases in higher saturated fatty acids (SFA), n-7 mono-unsaturated (MUFA), and n-3 polyunsaturated (PUFA), and a decrease in n-6 PUFA (**Table 1**.).

Table 1. Correlations of: a) total fatty acids, and b) individual fatty acids, with the clinical parameters of MS and lipid peroxidation indicator, after adjustment for age and BMI, in RRMS (n = 153) and PMS (n = 69) patients

	RRMS		**PMS**	
Fatty acids	**EDSS** **r** **p**	**MSSS** **r** **p**	**EDSS** **r** **p**	**MSSS** **r** **p**
Total SFAs	-0.06	-0.15	**0.36**	-0.01
	0.51	0.11	**0.005**	0.91
Total MUFAs	-0.09	-0.09	0.13	0.19
	0.32	0.31	0.32	0.16
Total n-6 PUFAs	0.12	0.15	-0.25	**-0.34**
	0.20	0.10	0.06	**0.009**
Total n-3 PUFAs	-0.04	-0.03	0.03	**0.35**
	0.69	0.74	0.82	**0.007**
Total n-6/n-3 ratio	0.03	0.04	-0.07	**-0.38**
	0.71	0.68	0.61	**0.003**

SFAs – saturated fatty acids; MUFAs – monounsaturated fatty acids; PUFAs-polyunsaturated fatty acids; EDSS – expanded disability status scale; MSSS – multiple sclerosis severity score; non-normally distributed data were log(2) transformed; r – Pearson's corre-lation coefficient; p-values < 0.05 were considered statistically significant.

2. Genetic analysis of functionally relevant variants in ferroptosis-related genes and variants associated with multiple sclerosis disease severity and target gene mRNA expression

Research of multiple sclerosis genetic risk factors was exponentially improved by genome wide association analysis (GWAS) approach, particularly in defining human leukocyte antigen (HLA) gene variants associated with disease onset. Still, the major challenge lies in resolving both, genetic and environmental factors related to MS disease severity and progression. By integrative approach and extensive datamining of multiple public data bases (FIVEx, GTEx, RegulomeDB, ENSEMBLE, HaploReg, GWAS Catalogue, GnomAD) and literature search we have selected 5 gene variants, which are proposed to be expression quantitative trait loci (eQTLs) for the top three differentially expression genes (DEGs) between mild relapse remitting (RR) and severe secondary progressive (SP) MS patients

published in our in previous paper (Stojkovic L, 2024): for *CDKN1A* rs3176326 and rs3176336, for *EGLN2* rs111833532 from *RAB4B-EGLN2* locus, and for *MAP1B* rs62363242 and rs1217817. In addition, two more gene variants from recent GWASs that have been associated with MS severity has been chosen and included in the study: rs10191329 from *DYSF–ZNF638* locus (5) and rs9643199 from *MTSS1* gene (6). Of note is that all of the patients included in the study were genotyped for the HLA-DRB1*15:01 Djuric et al., 2025, under review).

We analyzed if selected seven variants are associated with MS severity (604 RR MS/241 PMS) if they affect mRNAseq expression levels of top three DEGs (CDKN1A, MAP1B and EGLN2) (24 RRMS/24 PMS) in RRMS and SPMS patients and whether they are associated with the MS neurological deficit and severity parameters (EDSS, MSSS, gARMSS) (604 RR MS/241 PMS), taking into account the existing haplotypes. In addition, we analyzed their possible association with measured molecular components of the ferroptosis-related processes, such as lipid peroxidation and iron metabolism products, in plasma/serum of MS patients (153 RRMS/69 PMS).

- *MAP1B* rs62363242 rare A allele containing genotypes were associated with PMS course in females with an adjusted OR=1.56, 95%CI (1.06-2.29), p=0.02, independent of the HLA-DRB1*15:01 rs3135388 A allele presence.

- *CDKN1A* haplotypes inferred from rs3176326 G/A and rs3176336 A/T significantly affect *CDKN1* mRNA expression in both RRMS and SPMS.

- *RAB4B-EGLN2* rs111833532 in RRMS and rs10191329 from *DYSF-ZNF638* locus in male PMS patients showed significant association with the MS neurological deficit and severity parameters EDSS, MSSS and gARMSS

- *RAB4B-EGLN2* rs111833532 rare allele D containing genotypes (II vs. ID+DD) showed association with higher plasma 4-HNE in plasma of PMS patients. (Table 7). *MAP1B* rs62363242 rare allele A containing genotypes (GG vs. GA + AA) were significantly associated with iron metabolism parameters: with lower free iron (p=0.03) and higher transferrin (p=0.03) in serum of PMS patients. There was also a trend toward lower level of the third measured parameter, ferritin (p=0.08) within the same group of patients. *HLA-DRB1*15:01* rs3135388 risk A allele containing genotypes were significantly associated with higher levels of HEL (hexanoyl-lys adduct) in serum of MS patients overall (p=0.02) (Table 2).

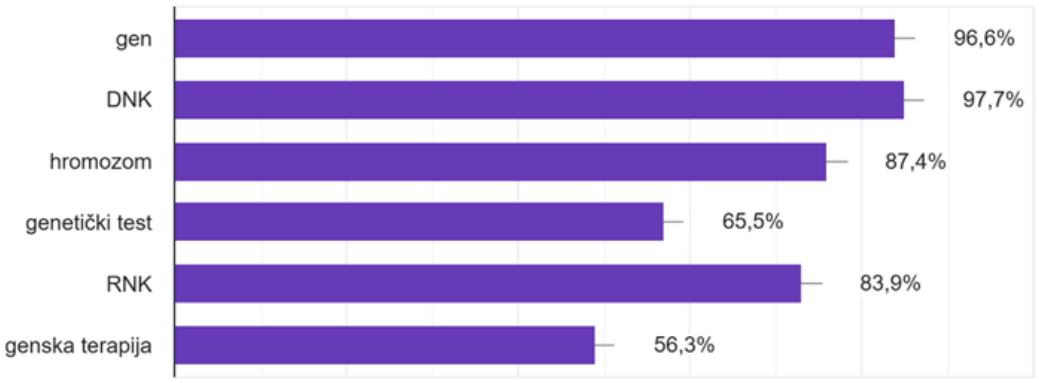
Table 2. Association of investigated gene variants with circulatory molecular indicators of processes associated with ferroptosis in MS patients.

Product of lipid peroxidation	RAB4B-EGLN2 rs111833532 (PMS)		p
	II	ID + DD	
4-HNE (pg/ml)	1299.98 ± 370.21	1938.05 ± 1540.60	0.04
	HLA-DRB1*15:01 (MS patients overall)		
	Without allele A	With allele A	
HEL (nmol/l)	12.51 ± 4.03	13.54 ± 3.63	0.018
Iron metabolism	MAP1B rs62363242 (PMS)		
	GG	GA + AA	
Iron (μmol/l)	15.91 ± 4.14	13.66 ± 4.78	0.03
Transferrin (g/L)	2.27 ± 0.37	2.53 ± 0.44	0.03
Ferritin (ng/ml)	72.25 ± 64.47	58.38± 73.62	0.08

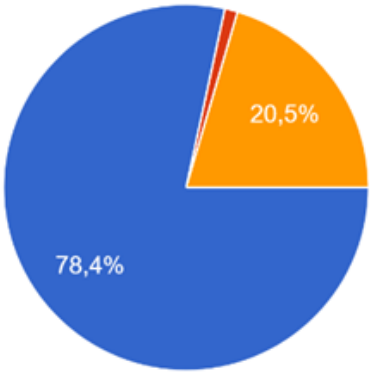
3. The second public survey "Science for Citizens: Genes and Multiple Sclerosis" Analytics

To provide the dissemination feedback from public and direct beneficiaries the Public Survey-Science for Citizens: Genes and Multiple Sclerosis has been created. The second survey that address basic knowledge about MS, genetics, therapy options and research related to MS in Serbia.was sent before the end of the project, which was year and a half after the first survey. The goal was to measure short and long term impact of the FerroReg project after the successfully implemented communication and dissemination activities with citizens and public beneficiaries. Participants of the second survey were 68.2% women, 41.4 % belonged to the 21-40 years age group, 35.6 % to the 41-60 years age group, 4.6 % older than 61 years and 18.4 % were younger than 20 years of age. 59.1% had high education and 56.8% were other profession than scientist or physicians, while 27.3 were students.

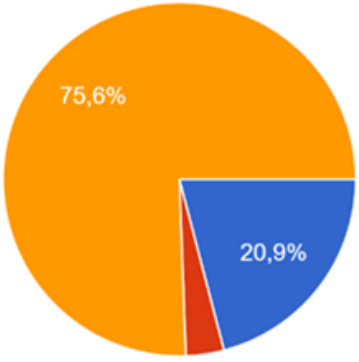
- In comparison with previous survey, participants of the second one have shown improved basic knowledge in genetics. Citizens are familiar with genetic terminology, almost 100% with terms "DNA" and "gene", followed by "chromosome" and "RNA".



- More participants (78.4%) are now aware that genetic testing can help with diagnosis, prevention and treatment of the disease. Only 1% thinks that genetic testing cannot help.



- Also, more of them (75.6%) know that the onset and progression of multiple sclerosis are influenced by a combination of genetic and environmental factors.



Main conclusions:

1. The differential relations according to disease course were established for the administration of immunomodulatory therapy, the occurrence of fatigue, or level of disability regarding the plasma/serum concentrations of lipid peroxidation products (MDA, 4-HNE, and HEL) and glutathione.
2. We suggest circulatory 4-HNE, a known product of lipid peroxidation, as an important parameter related to differences between RRMS and PMS.
3. No significant changes are found in the serum concentrations of iron metabolism indicators between the RRMS and PMS patients.
4. Erythrocyte profile of long-chain fatty acids could be specific for the course of MS, introducing the circulatory fatty acids as candidate molecular indicators for differentiating between the clinically defined RRMS and PMS.
5. Functionaly proposed genetic variants in ferroptosis related genes and those previously associated with disease severity are associated with progressive MS disease course, mRNAseq targeted gene expression, neurological deficit and severity parameters (EDSS, MSSS, gARMSS), plasma/serum concentrations of lipid peroxidation products (4-HNE, HEL), and iron metabolism indicators.
6. Almost 100% of citizens and public beneficiaries, filling the second Public Survey-Science for Citizens: Genes and Multiple Sclerosis are familiar with genetic terminology and more than 75% have the elementary knowledge of genetic role in complex diseases.

Strengths and Limitations

- Our study integrates genetic analysis and circulatory indicators of lipid peroxidation, which is the driver of ferroptosis, along with fatty acid profile between patients with RRMS and progressive MS with regard to clinical data and disease severity parameters, thus providing novel molecular markers, which can complement disease-related changes in the brain and undergo further research as potential therapeutic targets or supporting food supplementation. Since ferroptosis showed a substantial capacity during the last five years in several diseases, it is of great importance to define the state of the ferroptosis-related molecules in different phases and courses of MS. We have presented new original experimental data, in patients with RRMS and progressive MS and with regard to neurodegeneration.
- The main limitation of the current study is the lack of correlation of results with brain imaging. It would provide deeper insight into linking molecular changes in the periphery with disease activity in the brain and would strengthen the interpretation of the observed results and improve further selection of target molecules with regard to disease severity. However, in our future experimental work the effort will be made in this direction.
- A constant limitation is the impossibility of the inclusion of equal numbers of patients with mild and severe progressive MS disease course.

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5. https://ferroreg.vin.bg.ac.rs/public-survey-i/

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