Science Fund of the Republic of Serbia

FERROREG

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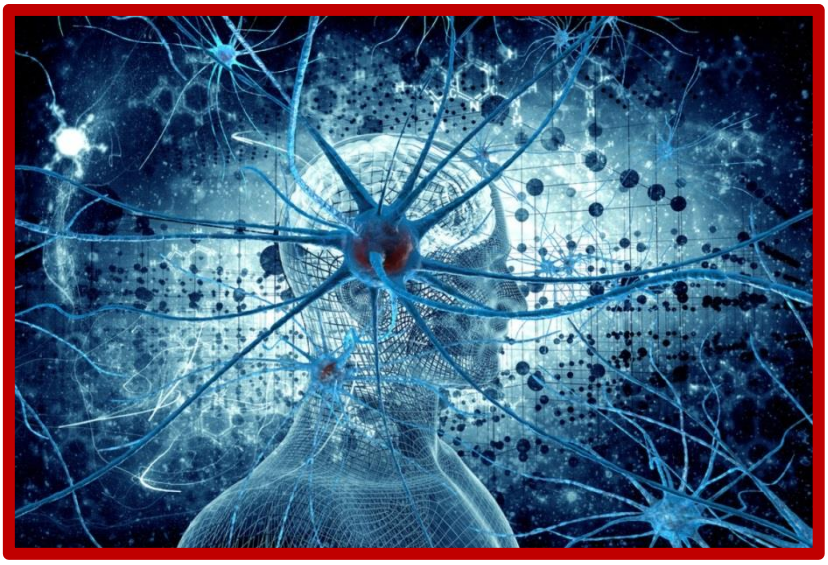
White Paper No1

Ferroptosis-related gene expression and essential trace elements profiles in differentiating multiple sclerosis phenotypes associated with disease severity

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



Science Fund of the Republic of Serbia

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Prepared on July, 20

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:

- The expression pattern/signature of a first comprehensive set of 138 ferroptosis-related genes in highly homogenous groups of patients with distinctive MS phenotypes, mild relapse-remitting (RR) and severe secondary progressive (SP) MS in peripheral blood mononuclear cells (PBMC). The panel includes genes with roles in lipid oxidative metabolism, antioxidant defense and iron metabolism, as well as their related main transcriptional regulators.
- In depth bioinformatical analysis of the protein–protein interaction and interplay of key differentially expressed genes (DEG) products, and their involvement in significantly enriched annotation terms.
- Assessed profiles and variations in levels of essential trace elements in sera of MS patients and controls based on demographic, disease course (RR, SP and primary progressive (PP)), and clinical parameters among MS patients.
- Analytics of a 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

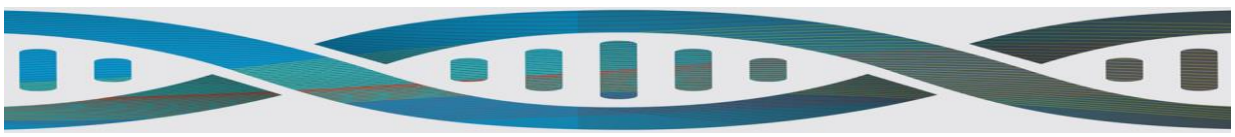
- **Scientific Research:** comprehensive elucidation of unknown ferroptosis regulatory patterns by experimentally gained results, research of existing databases, academic literature and by multidisciplinary approach.
- **Analytical Framework:** Using a blend of qualitative and quantitative methods, statistics 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. Targeted RNAseq Gene Expression Signature of Ferroptosis-Related Processes in patients with MS

We have determined and analyzed the expression pattern/signature of a comprehensive set of 138 ferroptosis-related genes in highly homogenous groups of 24 patients with RR (mild) and 24 SP MS phenotypes. The panel consists of genes with roles in lipid oxidative metabolism, antioxidant defense and iron metabolism, as well as their related main transcriptional regulators (**Figure 1**). The gene expression was determined by targeted RNAseq which has the highest accuracy among the next generation sequencing (NGS) techniques and represent cutting-edge technology in molecular biology (1).

Analysis of the data obtained from the targeted sequencing of 19 RR and 20 SP MS patients, retained after outlier removal, led to the identification of 26 DEGs according to nominal p value (**Table 1**). In the set of identified DEGs, 18 genes were upregulated while 8 genes were downregulated in SP patients compared to RR patients (1).



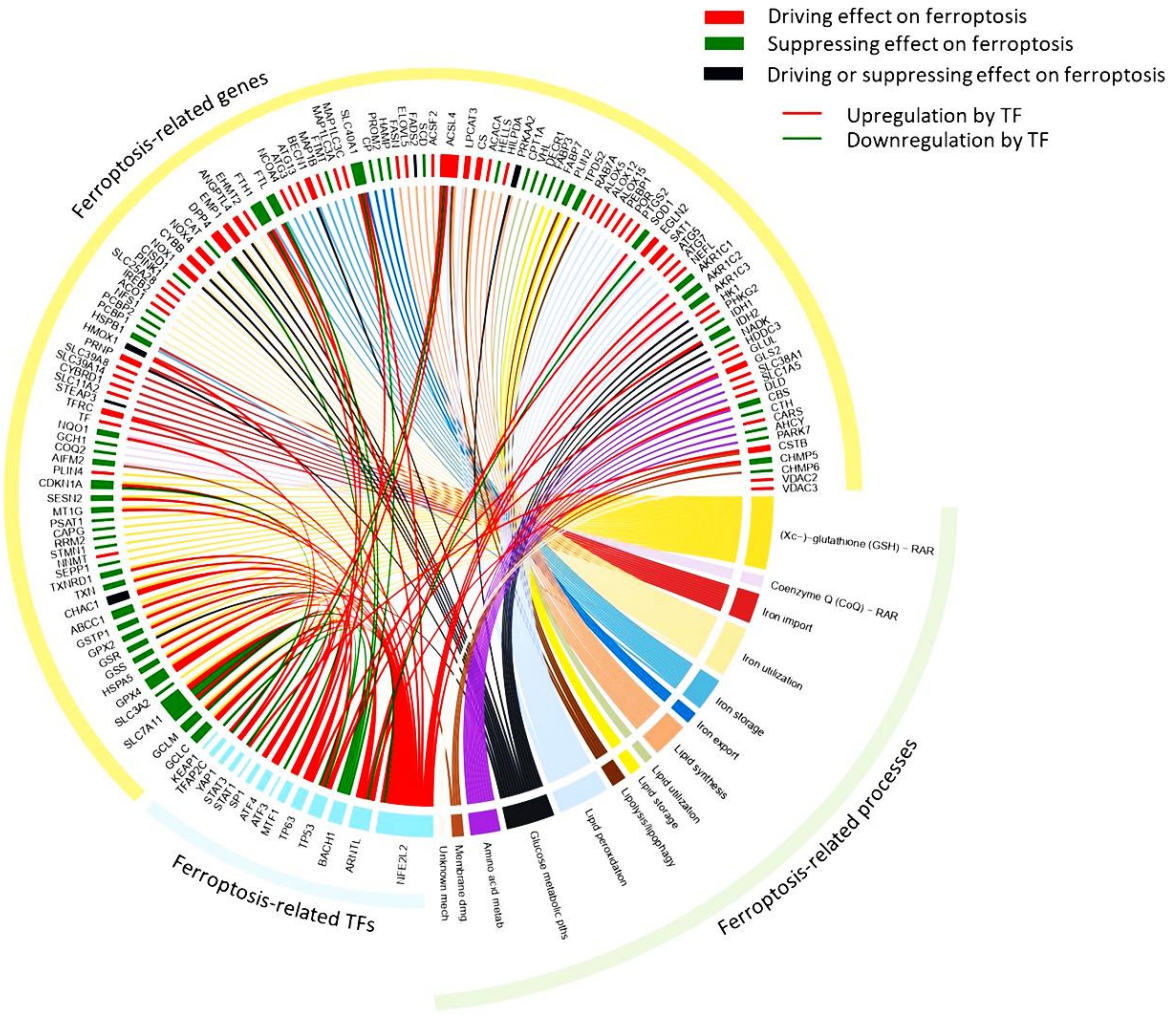


Figure 1. Circos plot of the ferroptosis-related genes selected for RNAseq panel. The Circos plot represents selected ferroptosis-related genes (yellow outer ribbon) with regard to the ferroptosis-related processes (green outer ribbon) and ferroptosis-related transcription factors (blue outer ribbon), which were included in the RNAseq panel. Within the inner circle, gene-associated rectangles are colored according to the proposed effect on ferroptosis: driving effect (red), suppressing effect (green), driving or suppressing (black). Rectangles associated with transcription factors are represented with a blue color. Rectangles associated with ferroptosis-related processes are represented with a unique color clearly distinguishing all of the presented processes. The size of rectangles in the inner circle depicts the number of associations within the Circos plot while the color of the line presents the link of the selected genes with the particular ferroptosis-related processes. Red and green lines depict upregulation and downregulation of selected genes by transcription factors, respectively.

The relative expression of the top three differentially expressed genes (CDKN1A, MAP1B and EGLN2), identified using RNAseq analysis, has confirmed the dysregulation of their mRNA levels in the independent replication group of 16 RR and 16 SP MS patients by quantitative Real Time PCR (qPCR) (1).

Table 1. Ferroptosis-related DEGs in SP compared to RR MS patients.

SP vs RR DEGs	baseMean	log2FoldChange	lfcSE	pvalue	padj
CDKN1A	9548.4098	0.591158406	0.114681	2.54E-07	3.24E-05
EGLN2	5756.6618	-0.338615318	0.067246	4.77E-07	3.24E-05
MAP1B	75.6888061	0.901324663	0.183941	9.58E-07	4.34E-05
SLC7A11	126.622192	0.606526575	0.132314	4.56E-06	0.000155
SAT1	19655.5985	0.4501215	0.099888	6.60E-06	0.000179
SLC11A2	1181.28541	-0.307764602	0.082231	0.000182	0.004127
CAT	4651.63507	0.29174057	0.081601	0.00035	0.006799
GLUL	3109.18502	0.380189011	0.11015	0.000557	0.009475
TP53	9275.04884	-0.23651102	0.075289	0.001682	0.025412
GCLC	3856.85128	0.226106063	0.073082	0.001976	0.02687
ALOX12	3420.79701	0.542998884	0.179849	0.002534	0.031335
NFS1	1626.194	-0.18163292	0.061148	0.002974	0.033708
EHMT2	325.41458	-0.146782661	0.053859	0.006424	0.067202
CYBB	22370.9217	0.299323864	0.113882	0.00858	0.083345
SEPP1	55.4441827	0.391031784	0.153583	0.010895	0.087159
GCH1	1363.03231	-0.285756188	0.111031	0.010063	0.087159
BECN1	7986.9329	0.110282249	0.043312	0.01089	0.087159
ATF3	438.943629	0.386548037	0.177873	0.029768	0.202423
CISD1	1126.71629	-0.193534746	0.088844	0.029378	0.202423
PLIN2	4488.7674	0.216134924	0.101398	0.033044	0.208698
ALOX5	7082.81376	0.221153845	0.104174	0.03376	0.208698
SLC40A1	13636.9118	0.250180235	0.119032	0.035571	0.210334
PSAT1	455.492259	-0.292751394	0.142061	0.039327	0.222852
EMP1	168.560458	0.275828894	0.13874	0.046801	0.2546
HSPA5#	3974.35589	0.198734257	0.063625	0.001787	0.010721
ATF4#	3565.62181	0.199836813	0.063727	0.001714	0.010721

SP vs RR DEGs – Differentially expressed ferroptosis-related genes in SP compared to RR MS patients; **baseMean** – average of the normalized count values, dividing by size factors, taken over all samples; **log2FoldChange** –indicates the gene expression changes in SP compared to RR MS samples on a logarithmic scale to base 2; **lfcSE** – the standard error estimate for the log2 fold change estimate; **pvalue** – P-value of the test for the gene; **padj** –adjusted P-value for multiple testing for the gene or transcript; **#** – DEGs obtained from the analysis of the sub-panel of highly expressed ferroptosis-related genes

2. Bioinformatic analysis

To investigate relationships between the identified DEGs, a protein–protein interaction (PPI) network was constructed. The zero-order network was created to keep only those DEGs coding for proteins that interact directly. Of the top three DEGs, two were also identified in the zero-order network (CDKN1A and MAP1B), forming an independent axis with a TP53 acting as a central hub molecule (Figure 2) (1).

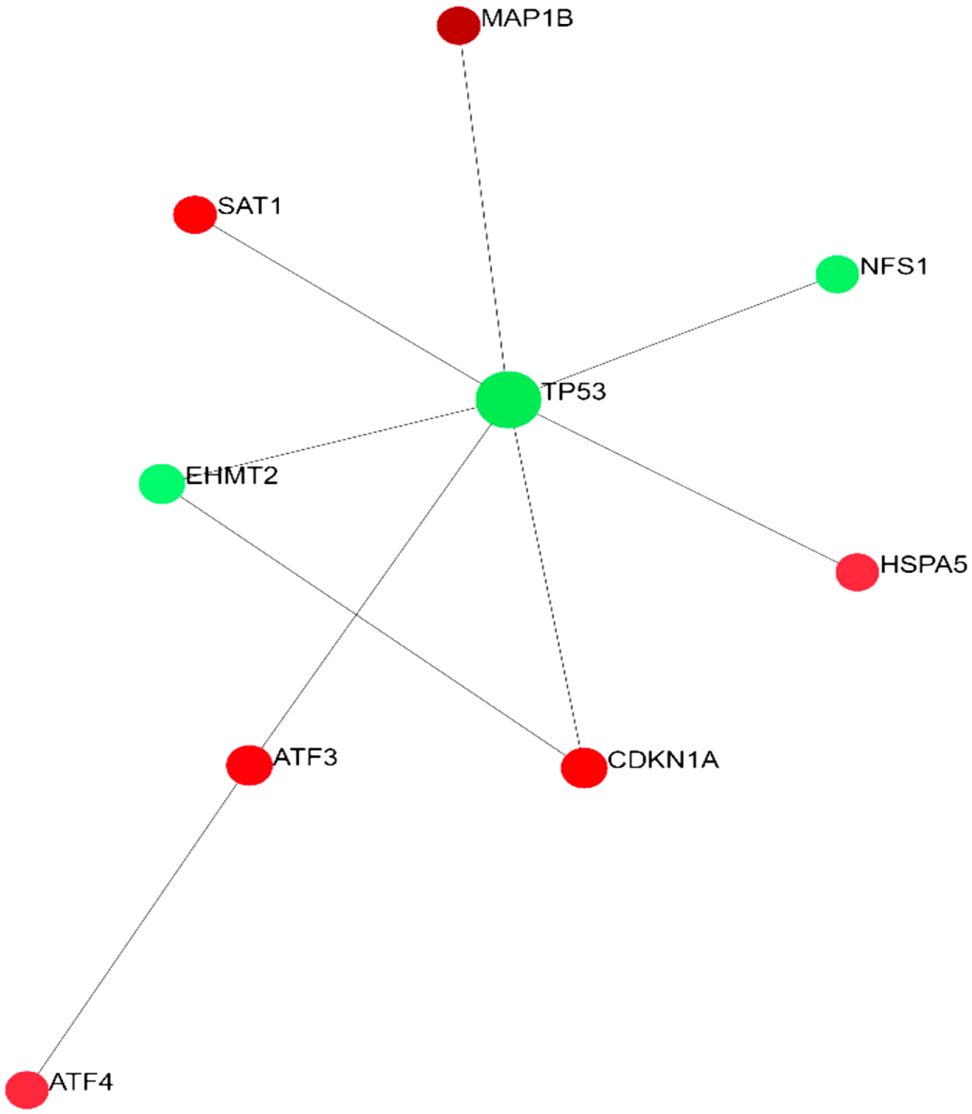


Figure 2. A zero-order PPI network of all ferroptosis-related DEGs between SP and RR MS patients. Green color represents downregulated genes while red color represents upregulated genes in SP compared to RR MS patients.

To understand the biological context of protein-protein interaction networks annotation enrichment analysis uses gene/protein annotations provided by knowledge-bases such as Gene Ontology (GO) or Reactome to infer which annotations are over-represented in a list of genes/proteins that can be taken from a network. Our further functional annotation analysis of the network has indicated significant enrichment of the ferroptosis pathway (KEGG), catalytic complex (Gene Ontology: Cellular Component) and iron (UniProt Annotated Keywords), which were employed for differential visualization of the network nodes (**Figure 3**) (1).

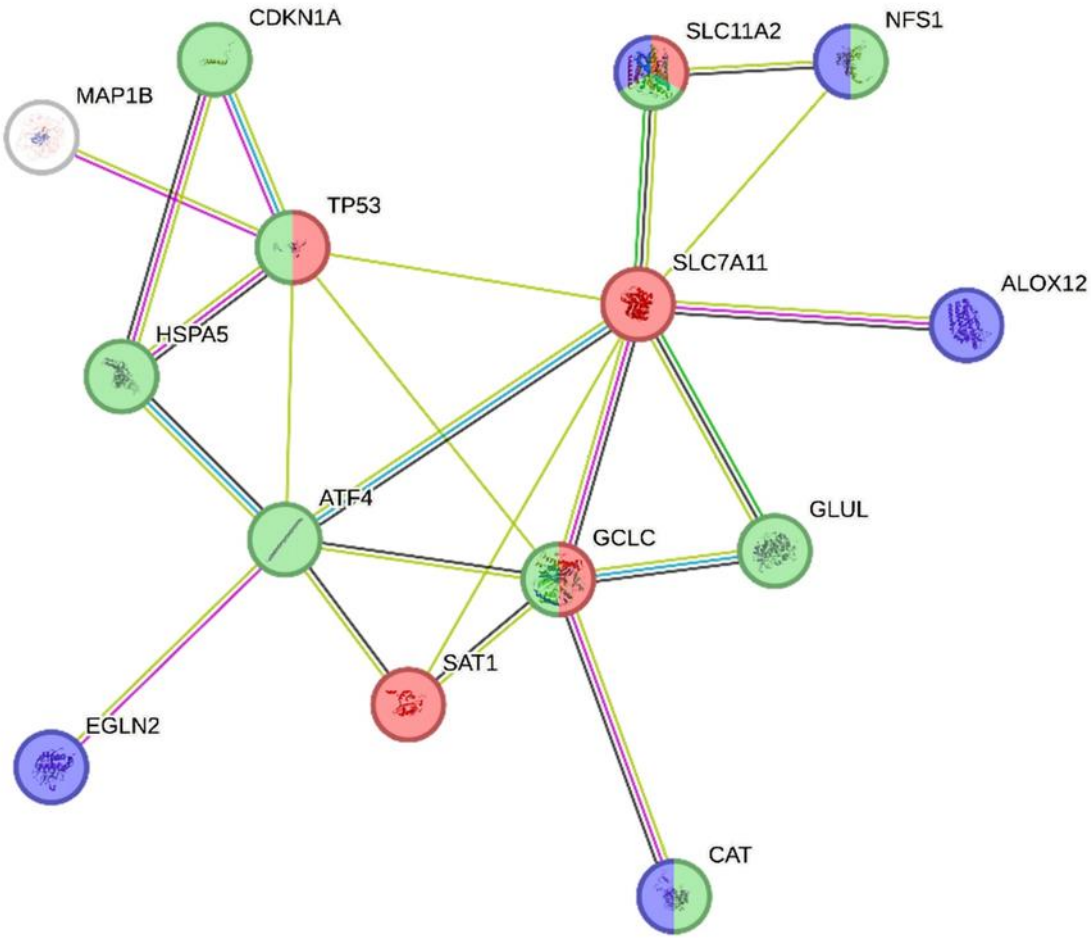


Figure 3. STRING PPI network of ferroptosis-related DEGs between SP and RR MS patients. The STRING v12 database was employed on a set of DEGs passing correction for multiple testing. Colors depict members of the top-enriched annotation terms in the network, computed by the STRING database. Red: ferroptosis (KEGG), Green: catalytic complex (Gene Ontology: Cellular Component), Blue: iron (Annotated key words UniProt). Edges represent protein–protein associations representing joint contributions to a shared function as described on https://string-db.org/.

3. Serum levels of essential trace elements

Since the levels of neurochemically important essential trace elements remain unclear in MS, particularly when looking at circulating levels across demographics and a characteristic of the disease itself we aimed to assess the levels of essential trace elements (Cr, Mn, Co, Cu, Zn, and Se) in the serum of MS patients compared to a matched control group and age between patients with different disease course thereby elucidating the prospective relevance of supplementing specific essential trace elements.

Neither MS patients nor controls were continuously supplemented with Cr, Mn, Co, Cu, Zn, and Se at least 6 months before collection of the samples. Compared to controls, Mn, Co, Cu, Zn, and Se levels were significantly decreased in patients with mild RR and severe SP MS

patients, while Cr levels in both groups were increased compared to controls. Se levels were significantly lower in severe SP MS compared to mild RR MS patients (**Table 2**) (2).

Table 2. Comparative Analysis of Serum Essential Trace Element Levels in Controls and Multiple Sclerosis Patients: Exploration across Control Group, mild RRMS and Severe SP MS

µg/L	**Controls n = 40**	**Mild RR MS patients n = 24**	**Severe SP MS patients n = 24**
Cr	3.340 ± 0.488	4.339 ± 1.177*	4.479 ± 1.056*
Mn	4.390 ± 2.672	0.454 ± 0.429*	0.528 ± 0.301*
Co	1.316 ± 0.630	0.536 ± 0.133*	0.515 ± 0.232*
Cu	908.358 ± 131.152	832.771 ± 172.688*	793.484 ± 143.797
Zn	1826.699 ± 1002.688	778.069 ± 314.677*	962.044 ± 425.765*
Se	86.706 ± 11.684	71.249 ± 18.425*	60.406 ± 14.625*N

Kruskal-Wallis ANOVA and Dunn's Post-Hoc test was used to seek differences between groups. Data are expressed as Mean ± SD. Significant difference at $P < 0.05$: * in respect to control; N in respect to mild RRMS patients. n – number of specimens for every particular element.

Further, we analyzed differences in trace elements with regard to different disease courses mild RR, severe SP and primary progressive PP MS patients. Co levels were significantly increased in PPMS compared to RRMS, and Cu levels were significantly increased in PPMS compared to SPMS (**Table 3**.)

Table 3. Serum Essential Trace Element Levels in Multiple Sclerosis Patients: A Comparative Analysis of Relapsing Remitting (RRMS), Secondary Progressive (SPMS), and Primary Progressive (PPMS) Multiple Sclerosis

µg/L	**RRMS n = 150**	**SPMS n = 52**	**PPMS n = 13**
Cr	4.808 ± 1.191	4.852 ± 1.364	4.699 ± 1.132
Mn	0.270 ± 0.274	0.332 ± 0.306	0.337 ± 0.300
Co	0.586 ± 0.249	0.494 ± 0.194	0.638 ± 0.189 A
Cu	855.711 ± 204.102	852.406 ± 195.148	1033.351 ± 186.050 B
Zn	709.336 ± 246.269	729.444 ± 244.258	724.508 ± 292.047
Se	72.428 ± 17.223	67.838 ± 18.820	67.517 ± 22.122

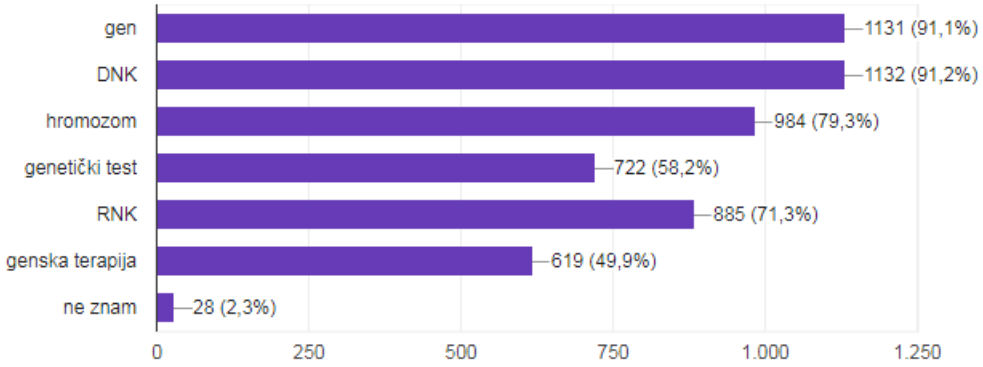
Kruskal-Wallis ANOVA and Post-Hoc Dunn's test were used to seek differences between groups. Data are expressed as Mean ± SD. A RR MS vs. PP MS progressive and B SP MS secondary progressive vs. PP MS. Significant difference at $P < 0.05$. n – number of patients.

When we analyzed MS patients overall compared to matched controls we found lower levels of Mn, Co, Zn, and Se, and higher level of Cr levels in serum of MS patients compared to the

control group. These trace elements not only effectively discriminate between MS patients and controls but also exhibit distinctive patterns among demographic subgroups.

4. Public survey "Science for Citizens: Genes and Multiple Sclerosis" Analytics

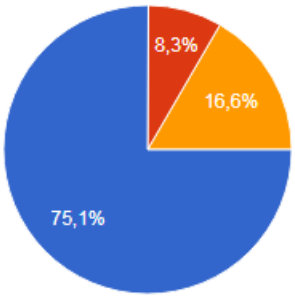
To provide the dissemination feedback from public and direct beneficiaries the Public Survey-Science for Citizens: Genes and Multiple Sclerosis has been created. The second survey is planned before the end of the project. One thousand two hundred and sixty one (1261) citizens have completed the questionnaire. 74.9% of them were women, 53.8 % belonged to the 41-60 years age group, 36.7 % belong to the 21-40 years age group, 8% older than 61 years and 1.2% were younger than 20 years of age. 61.6% had high education and more than 81% were not scientist or physicians (3).



Only 2.3% were not familiar with genetics or the terminology linked to it, while 5.8% didn't know that the genes could affect the development of the disease.

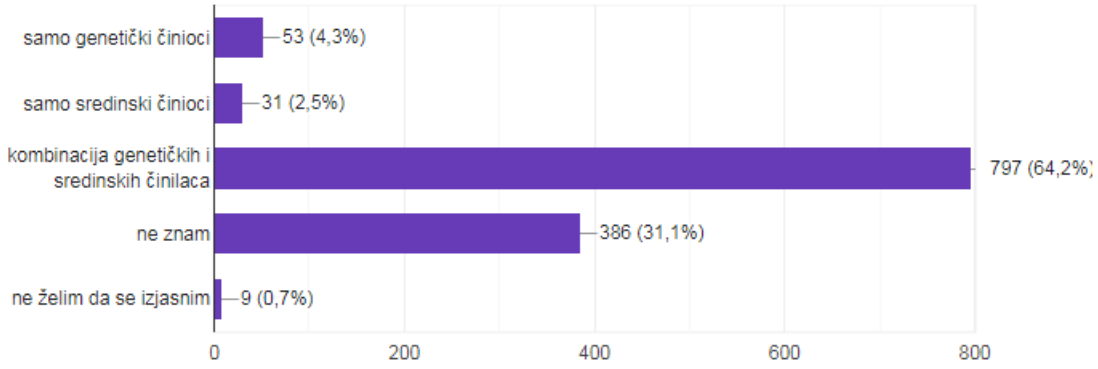
80.1 % would like to know if they have gene variants that affect the onset of complex diseases that arise from a combination of genes and lifestyle habits. Similar percentage of people (approximately 48%) prefer both, scientific paper and books from one side and Lectures from the other, as a way to find out more about genes and other factors that cause complex diseases, such as multiple sclerosis. Next are social networks 33.4%, YouTube 30.6%, followed by podcasts 27.2%, tv and radio 26.1%, while 3.9% are not

interested in this kind of knowledge. 78.5% would like to participate in the research by filling up the questioner about life style, food habits, exercise etc, but no more than half of them would like to participate in a research by giving a blood sample for DNA analysis even 62.7% are aware that data obtained during research are protected by the Personal Data Protection Act and that genetic information remains anonymous and would be used exclusively for scientific purposes.



75.1 percent are aware that genetic testing can help with diagnosis, prevention and treatment of the disease.

Fact that the onset and progression of multiple sclerosis are influenced by mutual effect of genes and environmental factors know 64.2%, while 31.1% are not aware of that.



The questions regarding the facts about multiple sclerosis as disease have been correctly answered by more than 50% of the examiners.

- 89.8% knew that multiple sclerosis is a chronic disease that affects the nervous system,
- 61.2% knew that it is more prevalent in women than man,
- 57.6% knew that the first symptoms of multiple sclerosis most commonly appear at younger age,

- 88.7% knew that multiple sclerosis causes muscle weakness, tremors, difficulty in walking and holding things in the hands, vision problems, difficulty to concentrate and cognitive processing, and
- 51.1% knew that vitamin D is an important factor in the prevention of multiple sclerosis.

Main conclusions:

1. The identified differentially expressed genes in PBMC between mild an severe MS patients were those that code for key components of the main ferroptosis-related processes, including:

- **cellular iron utilization and import/export:** NOX2/CYBB and SLC40A1 mRNA levels were upregulated and SLC11A2 downregulated in SP MS compared to RR MS patients
- **xC−/GPX4-dependent antioxidant defense system:** higher expression levels of SLC7A11 (xCT) and GCLC genes was found in SP MS patients
- genes known to regulate cell cycle, cell proliferation, apoptosis and **resilience to ferroptosis** in cells under stress: CDKN1A/p21 and MAP1B, HSPA5 and ATF4 were upregulated, while EGLN2 and TP53 were downregulated in SP MS patients compared to RR

2. Among differentially expressed genes the major hub between genes coding for proteins that interact directly was T53
3. Functional annotation enrichment analysis showed that the DEGs between SP and RR MS from our analysis are involved in ferroptosis pathway, catalytic cellular complexes and iron metabolism
4. Our findings underscore a substantive deficiency in Mn, Co, Zn, Se and increased levels of serum Cr in the MS patients overall compared to controls
5. Circulating Se levels could stratify cases of extreme MS severity, mild RR MS and severe SP MS
6. Circulating Mn levels showed a positive correlation with EDSS in SP MS patients

7. More than 50% of the 1261citizens and direct beneficiaries, filling the Public Survey-Science for Citizens: Genes and Multiple Sclerosis, have the elementary knowledge of genetic role in complex diseases and the basic facts about the multiple sclerosis.

Strengths and Limitations

- Our study integrates wide genetic signature related to ferroptosis and circulation trace elements in the easily obtainable PBMCs of MS patients with clinical data and disease severity, thus providing novel molecular markers, which can complement disease-related changes in the brain and undergo further research as potential therapeutic targets or supporting supplementation with trace elements. 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 mild RR and severe SP MS and with regard to neurodegeneration.
- The main limitation of the current study is the lack of correlation of genetic data 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 observed gene expression changes and improve further selection of target molecules with regard to disease modification. However, in our future experimental work the effort will be made in this direction.

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

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