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SEASONAL CHANGES IN SERUM METABOLITE AND CYTOKINE LEVELS IN MULTIPLE SCLEROSIS

Abstract: *multiple sclerosis (MS) is a chronic debilitating disease of unknown etiology. The disease has a seasonal exacerbation of clinical symptoms, which are frequently described in spring and summer. However, the mechanisms of such seasonal exacerbations remain unknown. In this study, we used targeted metabolomics analysis of serum samples using LC-MC/MC to determine seasonal changes in metabolites. We have found changes in multiple metabolites which differed depending on season. The ceramides, belonging to the sphingolipid pathway, were found activated in spring-summer (SS) and fall-winter (FW) MS, suggesting their central role in disease pathogenesis. Our identification of ceramide activation suggests a mechanism of neuron damage in MS which could be further investigated as therapeutic targets.*

Keywords: *multiple sclerosis, metabolites, season exacerbation, pathogenesis.*

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СЕЗОННЫЕ ИЗМЕНЕНИЯ УРОВНЯ МЕТАБОЛИТОВ И ЦИТОКИНОВ В СЫВОРОТКЕ КРОВИ ПРИ РАССЕЯННОМ СКЛЕРОЗЕ

Аннотация: рассеянный склероз (РС) – хроническое инвалидизирующее заболевание неизвестной этиологии. Заболевание характеризуется сезонными обострениями клинических симптомов, которые чаще проявляются весной и летом. Однако механизмы таких сезонных обострений остаются неизвестными. В данной работе мы использовали целевой метаболомный анализ образцов сыворотки с использованием LC-МС/МС для определения сезонных изменений метаболитов. Были выявлены изменения в нескольких метаболитах, которые различались в зависимости от сезона. Было обнаружено также, что церамиды, принадлежащие к сфинголипидному пути, активируются при весенне-летнем и осенне-зимнем РС, что позволяет предположить их центральную роль в патогенезе заболевания. Выявленная активация церамидов наводит на мысль о механизме повреждения нейронов при РС, который можно было бы далее исследовать в качестве терапевтической мишени.

Ключевые слова: рассеянный склероз, метаболиты, сезон обострения, патогенез.

Introduction: Multiple sclerosis (MS) is a disease presenting many challenges for healthcare providers. The disease presents in range of forms making diagnosis and prediction of disease progression difficult [1]. The most common form is RRMS, diagnosed in 85% of patients. This form is characterized by exacerbations followed by periods of complete remission. From the point of diagnosis, the course of MS can vary significantly between patients making early prediction of the clinical form of disease challenging [2]. This affects the selection of disease modifying therapies. Factors contributing to disease severity include sex and age of the patient, where old-

er males will have higher chance of poor prognosis [3]. Also, among the biomarkers, vitamin D level has been shown to have prognostic value at the early stages of the disease [4]. Study of the MS metabolome has demonstrated phosphatidylcholine proteins as potential biomarkers for MS diagnosis and prognostic markers for disease progression [5]. In another study, aberrant serum levels of kynurenic and quinolinic acids, metabolites of the kynurenine pathway, were shown to have a prognostic value in MS, specifically to determine the switch to progressive forms of the disease [6]. Still, application of identified biomarkers to diagnose and predict disease progression remains limited as there are still major gaps in understanding disease pathogenesis.

Seasonal biphasic pattern of MS relapses was demonstrated in an Italian cohort of patients [7]. Similarly, a seasonal pattern of relapse was demonstrated by Harding et al [8]. The contribution of seasons to the course of MS is a consistent feature of the disease and it has been suggested to include seasonality as a part of clinical trial design [9]. Although seasonal exacerbations are well documented, there is little known about biomarkers which could predict or explain them.

Season appears to be one of the factors contributing to disease exacerbation [9]. Therefore, in this study we stratified serum samples based on the season of collection. We have found that the ceramides, part of the sphingolipid pathway, were activated in spring-summer (SS) and fall-winter (FW) MS, suggesting their central role in the disease pathogenesis.

Materials and methods: Study subjects, samples: Serum samples from 33 (average age 34.2 ± 8.9 years, 11 male and 22 female) MS in remission admitted to the Republican Clinical Neurological Center, Republic of Tatarstan, Russian Federation were diagnosed based upon clinical presentation and brain MRI. Serum samples were collected from each patient as well as from 10 controls (average age 33.5 ± 11.5 years, 5 male and 5 female). Each patient and control provided a single serum sample. Samples were collected during the year and grouped based on three seasons: spring-summer (SS MS) (12 samples), fall-winter (FW MS) (21 samples).

Ethics statement: Informed consent was obtained from each subject according to the clinical and experimental research protocol, approved by the Biomedicine Ethic

Expert Committee of Republican Clinical Neurological Center, Republic of Tatarstan, Russian Federation (N: 218, 11.15.2012)

Metabolome analysis: Targeted metabolomics analysis including 36 pathways was done using LS-MS/MS analysis as described elsewhere [10] with modifications. In brief, serum from MS and controls was centrifuged (200×g; 10 min) and stored at -80°C before use. For the analysis, methanol extracts of serum were collected and used in LC-MS/MS. LC-MS/MS was done using high performance liquid chromatography (HPLC) Agilent 1260 Infinity II intracellular system combined with mass spectrometer tandem triple quadrupole mass spectrometer with linear ion trap (QTRAP 6500 plus (Sciex)) equipped with an electrospray ionization source ((ESI) Turbo V (Sciex)). Standards and heavy standards were purchased from Sigma and Toronto Research Chemicals [11]. The quality control (QC) samples contained a mixture of concentration-balanced standards according to plasma mean values. The QC samples were used as three technical replicates that were run three times before and after serum samples and extracted water samples prepared the same way as serum samples served as blanks.

Metabolites were separated during chromatography with hydrophilic interaction (HILIC) using a polymer-based column with amino functional group Asahipak NH2P-40 2E, 4 µm, 250×2 mm (Shodex). The chromatographic separation conditions were as follows: mobile phase A: 95% H₂O with 20 mM (NH₄)₂CO₃ and 5% acetonitrile, pH 9.8; mobile phase B: 100% acetonitrile. The gradient included: 0–3.5 min 95% B, 3.6–8 min 85% B, 8.1–13 min 75% B, 14–30 min 0% B, 31–41 min 95% B, stop 46 min. The speed of flow was 200 µL/min with the injected volume of 10 µL.

Metabolites were measured in positive and negative ionization modes by rapid polarity reversal. MRM (multiple reactions monitoring) transitions selection (pairs of parent ions and their daughter fragments) and mass spectrometry parameter optimization was performed by direct infusion using solution of standards. The obtained ion chromatograms were viewed using the Analyst 1.6.1 software package (Sciex). Chromatographic peaks were verified by comparison with retention times and MRM-ratio of internal standards labeled with stable isotopes, as previously described [10].

Serum metabolites amounts were estimated by integration of chromatographic peaks area using Sciex OS v1.4.018067 (Sciex) and manually confirmed. There were 340 of 452 metabolites identified and levels were measure in MS and control serum.

Statistical analysis: Metabolomic data were autoscaled (mean-centered and divided by the standard deviation of each variable) and the resulting scores analyzed by one-way ANOVA with pairwise comparisons and post hoc correction for multiple hypothesis testing using Fisher's least significant difference method in MetaboAnalyst 5.0 (<https://www.metaboanalyst.ca>, accession date 30.07.2021) [12].

The principal component analysis (PCA) was carried out using the R packages ggplot2 (version 3.3.3) and ggfortify (version 0.4.11) [13].

Results: MS seasonal groups: We had 33 MS samples as well as 10 controls collected during spring, summer, fall and winter. Since some of these groups had few samples, we have decided to combine samples collected in spring and summer as one group, identified as SS, while fall and winter season samples as FW. This resulted in a total of 12 MS and 5 controls in SS and 21 MS and 5 controls in FW groups. This separation is based on higher frequency of exacerbation of MS symptoms in spring and summer season as compared to fall and winter [8].

Metabolites analyses: PC analysis of metabolites in MS: PC analysis demonstrated differences in metabolites between seasonal controls and MS (Figure 1). It appears that the SS and FW controls clusters largely overlap with limited seasonal variations (Figure 1; red and green dots). In contrast, there was more clusterization between SS and FW MS as compared to seasonal controls. PC analysis revealed that some metabolites significantly vary depending on the season. This was evidently demonstrated when metabolites heatmap was analyzed (Figure 2). SS and FW control metabolite levels appeared similar with limited variation between seasons. In contrast, some SS and FW MS metabolites (ceramides, in particular) presented a cluster which differed significantly from that in control. There was also a distinct group of metabolites which was higher in SS or FW MS.

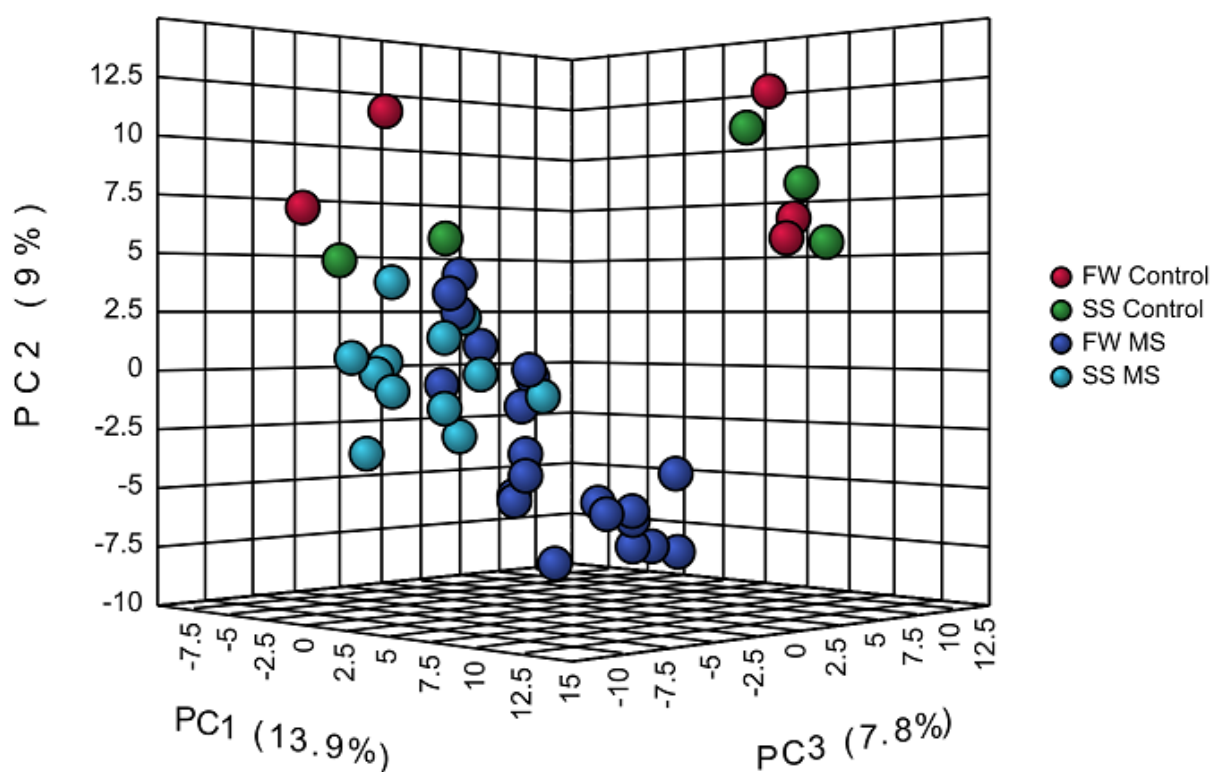


Figure 1. PCA of seasonal level of metabolites in MS and controls

This analysis is based on scaled levels of serum metabolites in MS and controls produced by MetaboAnalyst 5.0 (<https://www.metaboanalyst.ca>).

Analysis of metabolite levels in SS and FW MS: We have found levels of eight metabolites increased, while eighteen metabolites were decreased in SS MS as compared to related controls (Figure 2). There were 21 metabolites decreased and 18 metabolites increased in FW MS as compared to corresponding controls, which is a higher number than in SS MS (18 and 8, respectively). Also, the composition of decreased and increased metabolites in SS and FW MS differed. Out of 21 metabolites decreased in FW MS, 6 were also decreased only during this season and were not affected in SS MS. There was also a group of 3 metabolites decreased in SS MS, while unaffected in FW MS. Also, 11 metabolites were increased only in FW MS. One of the striking observations was that ceramides made a group which remained increased independent of the season when serum was collected. These results provide strong evidence of their central role in the disease pathogenesis.

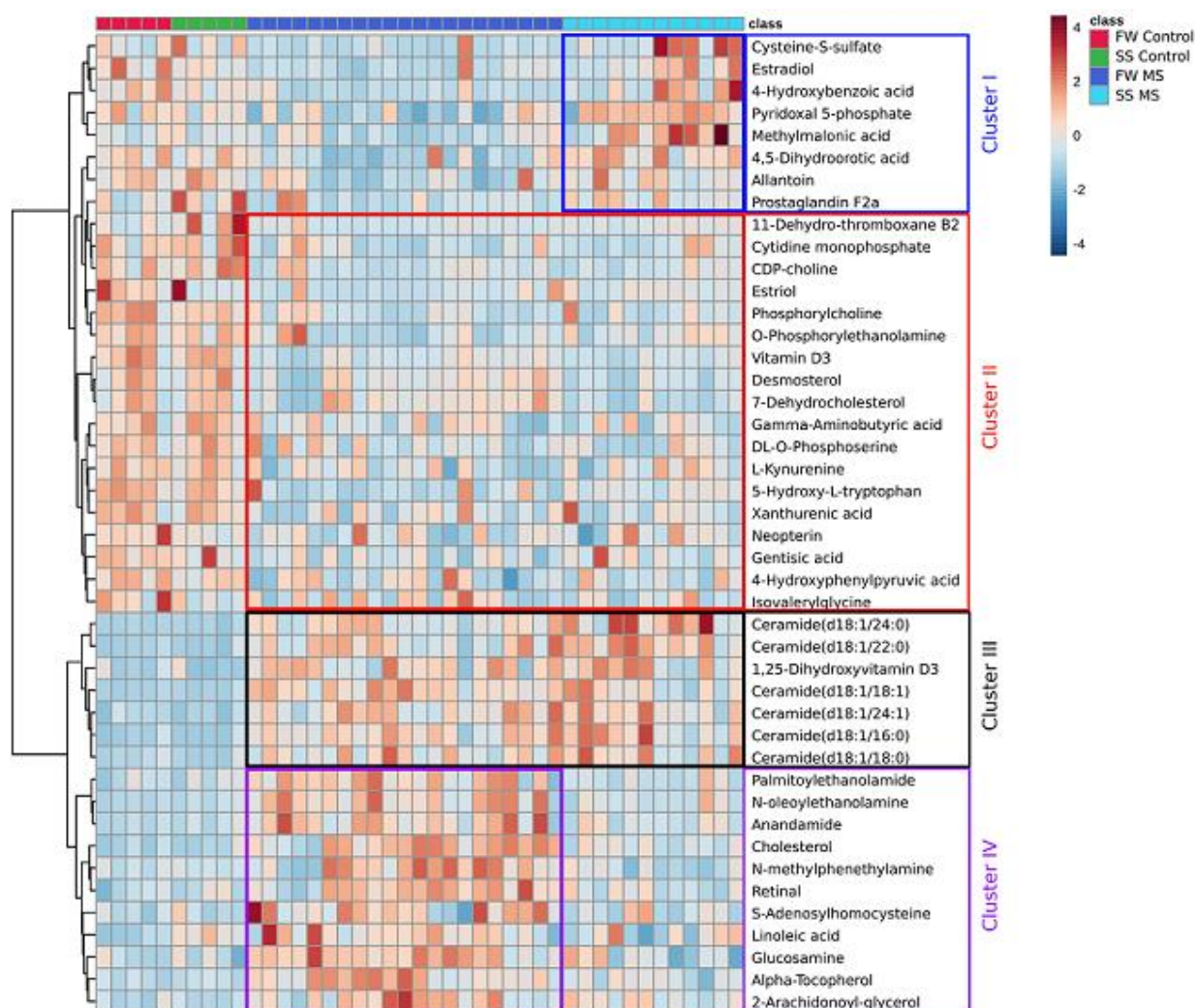


Figure 2. Heatmap analysis of the scaled metabolites levels

Heatmap analysis is produced using MetaboAnalyst 5.0 (<https://www.metaboanalyst.ca>). Euclidean distance method was used for clustering by metabolites.

All presented metabolites differed significantly between at least one comparison groups ($p \text{ adj.} < 0.05$, one-way ANOVA with Fisher's LSD)

Several clusters could be identified using heatmap analysis (Figure 2). Cluster I, containing eight metabolites increased in SS MS, while they mostly decreased in FW MS. In cluster II, metabolites demonstrated mostly similar changes in SS and FW MS, where some metabolites levels differ in individual patients not in the whole cohort. Cluster III contained seven metabolites mostly increased in SS and FW MS serum. It appears that the degree of upregulation was slightly higher in SS MS than in FW MS. Interestingly, this cluster contained all ceramide metabolites identified af-

ected in MS. The cluster IV included metabolites increased in FW MS as compared to SS MS. When metabolite levels were analyzed between different seasons, FW MS was characterized by increased levels of 13 metabolites, while five were increased in SS MS (Figure 4). It is an interesting observation as it appears that metabolic activity is in FW MS remains high.

Discussion: We have identified multiple metabolites differentially activated in SS and FW MS. These differences appear to be significant, and, potentially, contributing to seasonal exacerbation of the disease. Our data confirms previous findings of an increased level of ceramides in MS [14]. Moreover, our data supports Kurz et al suggestion on role of ceramides in MS pathogenesis [15].

Ceramides are lipid molecules containing a sphingoid long-chain base linked to an acyl chain using as an amide bond [16]. In neurons, ceramides are accumulated on soma and axon contributing to the neuronal adhesion, modulation of ion channels and neurotransmitter receptors expression [17]. However, an increased level of ceramides could lead to the neuronal apoptosis and death due to the oxidative stress [18]. This oxidative stress could be induced by virus proteins and pro-inflammatory cytokines [19].

Our data supports the previous observations demonstrating low levels of vitamin D in MS [20]. Our data further advances our understanding of the role of vitamin D and its derivatives in MS pathogenesis. Concurring with published data, levels of vitamin D were lower, while contrasting, levels of calcitriol (1,25-Dihydroxyvitamin D₃) were higher in MS as compared to seasonal controls. Calcitriol is an active form of this vitamin, produced in the kidney through a second hydroxylation of vitamin D [20]. Our results suggest that this enzymatic transformation of vitamin D is increased in MS, which may happen in the kidney. It has been demonstrated that vitamin D deficiency could lead to lowering blood calcium level due to decreased intestinal absorption [21]. That could signal the parathyroid glands to increase the secretion of parathyroid hormone activating renal mitochondrial 1- α -hydroxylation of vitamin D producing active calcitriol [22]. As a result, vitamin D deficiency could lead to a secondary hyperparathyroidism. This secondary hyperparathyroidism was described in

MS [23]. Our data could provide some explanation to the inconsistent results of therapeutic use of vitamin D [24], as the active form of this vitamin is already at the high level in these patients. Still, low levels of vitamin D are consistently found in MS, regardless of the season, indicating its role in the disease pathogenesis.

Our data identified seasonal changes where levels of metabolites and inflammatory mediators were affected in SS and FW MS. We have found that the ceramides, part of the sphingolipid pathway, were activated in SS and FW MS, suggesting their central role in the disease pathogenesis.

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