

# Short and Medium Chain Fatty Acids in a Cohort of Naïve Multiple Sclerosis Patients: Pre- and Post-Interferon Beta Treatment Assessment

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**Introduction:** Alterations in intestinal permeability and microbiota dysregulation have been linked to the development of multiple sclerosis (MS). Short-chain fatty acids (SCFA) and medium-chain fatty acids (MCFA) are products of gut bacteria fermentation which are involved in immune regulation processes. In MS, SCFA have important immunomodulatory properties both in the periphery and the central compartment. Interferon  $\beta$  (IFN $\beta$ ) was the first disease-modifying therapy approved for the treatment of MS and its effects on the gut microbiota are not fully elucidated.

**Patients and Methods:** We performed a prospective observational study aimed to assess peripheral levels of SCFA and MCFA in 23 newly diagnosed, treatment-naïve MS patients (nMS) before and after one year of IFN $\beta$  treatment and 23 healthy controls (HC). We investigated their associations with inflammation, interleukin-10 (IL-10), and blood-brain barrier permeability, matrix metalloproteinase 9 (MMP9).

**Results:** No significant differences in SCFA/MCFA levels were observed between baseline and after IFN $\beta$  treatment. Caproic acid levels were significantly higher in nMS compared to HC (1.64 vs 1.27  $\mu$ M,  $p=0.005$ ). The butyric acid/caproic acid ratio was higher in HC compared to nMS (5.47 vs 2.55,  $p=0.005$ ). Correlation analysis revealed associations between SCFA/MCFA levels and inflammatory biomarkers.

**Conclusion:** nMS have a higher gut-inflammatory activity as seen by the caproic acid ratio as opposed to HC. In this cohort, IFN $\beta$  does not appear to modify the peripheral SCFA/MCFA levels after one year of treatment. The quantifications of peripheral SCFA/MCFA may prove to be a useful biomarker for gut-brain axis disruption in MS patients.

**Keywords:** butyric acid, propionic acid, acetic acid, caproic acid, cytokine, gut microbiota, matrix metalloproteinase

## Introduction

Multiple sclerosis (MS) is a chronic autoimmune disease affecting the central nervous system (CNS), characterized by chronic inflammation and neurodegeneration, leading to demyelination and axonal loss.<sup>1,2</sup> It represents an important cause of neurological handicap in young adults,<sup>3</sup> with more than 2.8 million individuals affected worldwide and an increased prevalence in the past decade.<sup>4,5</sup> This can be explained by the increase in life expectancy, the growth of the adult population and improved data collection methods with national MS registries.<sup>6</sup>

The hygiene hypothesis<sup>7</sup> states that the rise in autoimmune diseases like MS, particularly in industrialized nations, stems from an underdeveloped immune system during formative years.<sup>8</sup> Despite the hygiene hypothesis not elucidating the risk of MS, it prompted the investigation of the gut microbiota in MS. In healthy individuals, within the gut

environment, commensal and anti-inflammatory bacteria engage with the endothelium of the intestinal barrier to uphold homeostasis.<sup>9</sup> These bacteria ferment dietary fibers to produce short-chain fatty acids (SCFAs) such as acetic acid (AA), propionic acid (PA) and butyric acid (BA).<sup>10</sup> SCFAs have immunomodulatory roles, promoting the differentiation of regulatory T cells (Tregs) and interleukin-10 (IL) secretion while controlling the populations of pro-inflammatory Th cells.<sup>11,12</sup> Dysbiosis disrupts this balance, favoring the differentiation of Th1/Th17 lineages and perpetuating peripheral and central inflammation.<sup>13</sup>

Some SCFA have the ability to penetrate the blood-brain barrier (BBB) and enter the CNS, where they play immunomodulatory roles by activating specific G protein-coupled receptors (GPCRs) and inhibiting the histone deacetylase activity (HDAC-i).<sup>12,14</sup> Unlike SCFA, medium-chain fatty acids (MCFA) such as caproic acid (CA) were demonstrated to promote inflammatory processes by augmenting Th1/Th17 lymphocytic populations and their by-products.<sup>15</sup> SCFA and MCFA have broad regulatory roles as signaling molecules in immune modulation, actively participating in energy metabolism, acting as “metabokines” in physiological processes, as proposed by Yu et al.<sup>16</sup>

The dysfunction of the intestinal barrier, particularly in the context of dysbiosis and intestinal inflammation, leads to increased permeability and the maintenance of systemic inflammation.<sup>17</sup> Subsequently, the disruption of the BBB allows inflammatory mediators to enter the CNS, contributing to demyelination and neurodegeneration.<sup>18</sup> Matrix metalloproteinases (MMPs), are a family of enzymes involved in the breakdown and remodelling of extracellular matrices. MMP9 demonstrated to carry intense pro-inflammatory roles, with a highly elevated expression in gut inflammatory conditions and in MS.<sup>19–21</sup>

Interferon beta (IFN $\beta$ ) was the first disease-modifying therapy (DMT) developed for the treatment of MS.<sup>22</sup> The effects of IFN $\beta$  on the gut microbiome are unclear, as IFN $\beta$  interferes with antigen presentation, thereby inducing a shift from Th1 to Th2 cytokine expression<sup>23</sup> and may influence the microbiome composition.<sup>24</sup>

## Materials and Methods

### Objectives

To evaluate the peripheral levels of SCFA: AA, PA, and BA and MCFA: CA, in a group of treatment-naïve RRMS patients (nMS) from the perspective of inflammation (IL-10), and BBB permeability (MMP9) and to compare the peripheral levels of SCFA/MCFA after one year of IFN $\beta$  treatment.

### Study Participants

We conducted a prospective, non-interventional, observational study that enrolled 28 consecutive newly diagnosed, nMS patients who presented to our clinic between 2022 and 2023, eligible for IFN $\beta$  treatment.<sup>25</sup> Inclusion criteria comprised: (1) disease onset < 1 year before inclusion; (2) diagnosis of RRMS according to the 2017 McDonald criteria;<sup>26</sup> (3) treatment-naïve status; (4) eligibility for IFN $\beta$  therapy initiation; (5) age between 18 and 60 years; (6) provision of informed consent for study participation; (7) agreement to abstain from alcohol consumption for 1 week before blood sampling; (8) ensured a normal night's sleep (minimum 6 hours) before blood sampling; (9) good tolerability of the IFN $\beta$  administration. Exclusion criteria were: (1) presence of other diagnosed autoimmune disorders, celiac disease or neoplasms; (2) concurrent use of pro- or prebiotics; (3) glucocorticoid or antibiotic therapy in the month preceding blood sampling. The eligibility for IFN $\beta$  initiation consisted of the following: (1) RRMS diagnosis based on McDonald 2017 criteria;<sup>26</sup> (2) no spinal cord lesions; (3) mild lesion burden; (4) patient preference. During the follow-up period, 5 nMS patients were excluded from the study analysis: three patients experienced adverse reactions to IFN $\beta$  therapy (one with a dermatological reaction, two with elevated liver enzymes), and 2 patients exhibited disease activity after initiating IFN $\beta$  treatment, leading to the escalation of the treatment regimen. Therefore, the study included 23 nMS patients who were evaluated at baseline and after one year of IFN $\beta$  therapy and 23 hC age, gender, and approximate body-mass index (BMI) matched and from the same geographic area.

For HC subjects, any exclusion criteria included: (1) recent alcohol consumption (1 week) before blood sampling; (2) history of autoimmune or neoplastic disorders; (3) inadequate sleep the night before blood serum sampling (less than 6 hours); (4) concurrent use of pro- or prebiotics; (5) antibiotic therapy in the month preceding blood sampling;

All nMS patients underwent clinical evaluations at baseline, 3, 6 and 9 months (as per internal National MS program protocol), and one year after initiating IFN $\beta$  therapy. The clinical evaluation was conducted by an EDSS-certified MS neurologist (authors). Socio-demographical data and BMI were noted in the patient's chart at baseline and after 1 year. Disability was assessed using the Expanded Disability Status Score (EDSS) at baseline (EDSS<sub>0</sub>) and at one year (EDSS<sub>1</sub>). The absolute number of relapses before treatment initiation was noted as R (including the onset as a relapse for statistical purposes). Baseline brain and spinal cord magnetic resonance imaging (MRI) scans were obtained for all nMS patients as part of the National MS program protocol.

All participants provided informed consent. The study was approved by the Ethics Committee of the Emergency Clinical County Hospital Targu Mures (Approval No. 13555/21.06.2022) and the Ethics Committee of the University of Medicine, Pharmacy, Science and Technology "George Emil Palade" of Targu Mures (Approval No. 1832/14.07.2022).

## Collection of Blood Samples

For the nMS patients venous peripheral blood samples were collected at baseline and after one year of treatment with IFN $\beta$  therapy in the morning, between 8:00 and 10:00 A.M., after fasting for at least 12 hours, using two clot accelerator tubes. After sitting at room temperature for 20 minutes, the tubes were centrifuged at 3500 rotations per minute for 20 minutes. The serum was then separated and aliquots were stored at  $-70^{\circ}\text{C}$  until further processing. At baseline, the samples were collected for SCFA/MCFA analysis, IL-10 and MMP9. After one year of IFN $\beta$  treatment venous peripheral blood was harvested for the second SCFA/MCFA analysis (the patients were asked to come after at least 24 hours after their IFN $\beta$  injection, based on their administration protocol). For the HC, venous peripheral blood samples were collected after the MS group was established and included SCFA/MCFA analysis, and MMP9.

## Sample Analysis

### Cytokines and MMP9 Immunoassays

All immunoassays were performed at the Humoral Immunology Laboratory of the Center for Advanced Medical and Pharmaceutical Research (Targu Mures, Romania). Before processing, serum aliquots were transported on ice, thawed, and vigorously mixed. For cytokines and MMP9, samples were processed using commercially available multiplex bead-based kits, following the manufacturer's instructions: MILLIPLEX<sup>®</sup> Human Th17 Magnetic Bead Panel kit (Merck-Millipore, catalogue number HTH17MAG-14K) and MILLIPLEX<sup>®</sup> Human MMP Magnetic Bead Panel 2 kit (Merck-Millipore, catalogue number HMMP2MAG-55K, for MMP9), respectively. The cytokines of interest for the analysis were IL-10, IL-17A/IL-17F. IL-17A-17F was below the limit of detection and could not be quantified in the present cohort. The IL-10 levels were performed only in nMS patients and not in HC. The values were reported in ng/mL. Analysis was performed using Luminex<sup>®</sup> xMAP<sup>®</sup> technology on a properly calibrated FLEXMAP 3D<sup>®</sup> analyzer (Luminex Corporation, Austin, TX, USA) and xPONENT<sup>®</sup> software version 4.3 was used for data acquisition. Results are reported in ng/mL.

### SCFA/MCFA Analysis

Analysis of plasmatic SCFA/MCFA concentration levels was performed at the Chromatography and Mass Spectrometry Laboratory of the Center for Advanced Medical and Pharmaceutical Research (Targu Mures, Romania) using a validated liquid chromatography–tandem mass spectrometry (LC-MS/MS) method developed in-house. The LC-MS/MS system used was composed an AB Sciex (Framingham, USA) 4600 TripleTOF type mass spectrometer coupled with a Perkin Elmer (Waltham, USA) Flexar FX-10 ultra-high performance liquid chromatograph (UHPLC). Vortex mixers used in the study were manufactured by Velp Scientifica (Usmate Velate, Italy) and Heidolph (Schwabach, Germany), while centrifugation was performed on an Eppendorf (Hamburg, Germany) centrifuge. Other equipment used for sample preparation were Radwag (Radom, Poland) XA 523Y analytical balance, JP Selecta (Barcelona, Spain) ultrasonic bath, Eppendorf (Hamburg, Germany) automatic pipettes and a Millipore (Burlington, USA) DQ3 water ultra purifying system.

After chemical derivatization with 3-nitrophenylhydrazine, SCFA/MCFA (AA, PA, BA, and CA) were separated chromatographically within 18 minutes run-time, using isocratic elution with mobile phase consisting of 0.2% formic acid and acetonitrile with a constant flow rate of 0.5 mL/minute on a Phenomenex Gemini NX-C18 column (column size 3.0×100 mm, particle size 3 µm). The analytical column was thermostated at 25 °C, while the samples were kept in the autosampler at 20 °C. The sample injection volume was 10 µL. After separation on chromatographic column, the analytes and internal standard were ionized for mass spectrometric detection using negative electrospray ionization mode (ESI<sup>-</sup>). Detection was performed by monitoring specific fragmentation patterns for each analyte and for the internal standard in MS/MS multiple reaction monitoring (MRM) mode. The parameters for the ionization source were as follows: −4500 V, vaporizer temperature: 500 °C, Ion Gas Source 1: 20 bar, Ion Gas Source 2: 25 bar, Curtain Gas: 30 bar, Declustering Potential: −50 V, Ion Release Delay: 39 ms, Ion Release Width: 17. For quantification, specific fragments were summed to increase peak intensity, and thus sensitivity of the method. Calibration curves were obtained using certified reference substances, with a concentration range of 10–1000 ng/mL for each analyte, and were constructed with calibration standards at six different concentration levels using linear fit and 1/y<sup>2</sup> weighting. Before LC-MS/MS analysis, the calibration standard solutions containing all analytes and the internal standard underwent a chemical derivatization reaction. Similarly to calibration standard solutions, patient plasma samples were also derivatized before LC-MS/MS analysis. The fragments used for quantification, arranged by intensity, along with retention times of analytes are described in Table 1.

## Statistical Analysis

The clinical and demographical characteristics of the study participants were assessed using descriptive statistics. Continuous variables were summarized using either the mean and standard deviation (SD) or median with the associated 95% confidence interval (95% CI). The choice between the mean/SD and median [95% CI] was based on the empirical distribution to the normal probability distribution. Before data analysis, normality was evaluated using Shapiro Wilk, quantile plots (Q–Q) as well as skewness and kurtosis. For data comparison, we used parametric and non-parametric tests depending on whether the data was paired or unpaired (Wilcoxon matched-pairs signed rank test, Unpaired *T*-Test with Welch's correction or Mann Whitney U). We then analysed the ratio BA/CA in nMS vs HC. To assess the associations between the SCFA levels and the inflammatory biomarkers, we performed Spearman correlation analysis. Additionally, we conducted a linear regression analysis to explore the relationship between the analysed factors. The significance level was set at  $p < 0.05$  for all analyses. Statistical analysis was conducted using SPSS v26 statistical computing software and GraphPad Prism 9 for graphical purposes.

## Results

### General Characteristics of the Study Population

The general clinical and socio-demographical characteristics of the study participants are presented in Table 2. None of the study participants had relapses during the first year of treatment.

**Table 1** Mass Spectrometric Fragments Monitored for Analyte Quantification

SCFA	Molecular Weight (g/mol)	Parent Ion (m/z)	Fragment Ions (m/z)	Collision Energy (V)	Retention Time (min)
AA	60	194.07	152.05; 137.05; 122.02; 178.07; 150.05	−20	2.2
PA	74	208.09	152.05; 137.05; 150.05	−20	2.7
BA	88	222.07	152.05; 137.05; 122.02; 178.07; 150.05	−20	4.0
CA	116	250.13	152.05; 137.05; 178.07	−20	13.0
Caproic-d3 acid (internal standard)	119	253.14	155.05; 140.05; 181.07	−20	13.0

**Abbreviations:** SCFA, short chain fatty acids; AA, acetic acid; PA, propionic acid; BA, butyric acid; CA, caproic acid.

**Table 2** Clinical and Socio-Demographical Characteristics of the Study Participants

Variable	nMS (n=23)	HC
<b>Environment (Urban: Rural)</b>	13:10	13:10
<b>Gender (F:M)</b>	14:9	14:9
	<b>Median</b>	<b>95% CI</b>
<b>Age at study inclusion (years) <sup>¥</sup></b>	31 [25; 36]	30 [25; 35]
<b>Age at MS onset (years) <sup>¥</sup></b>	30 [24; 34]	
<b>Onset to treatment (months) <sup>¥</sup></b>	6 [4; 11]	
<b>Onset to diagnosis (months) <sup>¥</sup></b>	3 [1; 4]	
<b>Diagnosis to treatment (months) <sup>¥</sup></b>	3 [1; 4]	
<b>R <sup>¥</sup></b>	1 [1; 2]	
<b>EDSS_0 <sup>¥</sup></b>	1 [1; 2]	
<b>EDSS_1 <sup>¥</sup></b>	1 [1; 2]	
<b>T2 lesions (baseline)</b>	11.5 [7; 14]	
<b>BMI_0</b>	25.71 [24.77; 27.68]	25.34 [24.31; 27.02]
<b>BMI_1</b>	25.39 [24.17; 26.96]	

**Abbreviations:** BMI, body mass index; EDSS\_0, Expanded disability status score at inclusion; EDSS\_1, Expanded disability status score after one year of treatment; F, female; M, male; R, relapses before treatment; p <sup>¥</sup>, non-parametric distribution based on Shapiro test of normality.

## IFN $\beta$ Does Not Modify the Peripheral Levels of SCFAs/MCFA in nMS Patients at Baseline and After One Year of IFN $\beta$ Treatment

The data shows a decrease in the median levels of all the SCFAs and the BA/CA ratio, and a slight increase in the levels of the CA after one year of IFN $\beta$  treatment compared to baseline in nMS patients, however, the results are not statistically significant (Wilcoxon matched-pairs signed rank test) (Table 3). Data is represented in Figure 1 for observational purposes as median with 95% CI.

## Levels of Biomarkers Differ from nMS and HC

At baseline, MMP9 levels were markedly elevated in nMS patients compared to HC ( $p < 0.001$ ). No significant difference was observed in the levels of AA, PA and BA in nMS vs HC. However, CA levels were significantly higher in nMS patients compared to HC ( $p = 0.005$ ). The BA/CA ratio was significantly lower in nMS patients compared to HC ( $p = 0.005$ ) (Table 4). Data is presented in Figure 2 for demonstrational purposes.

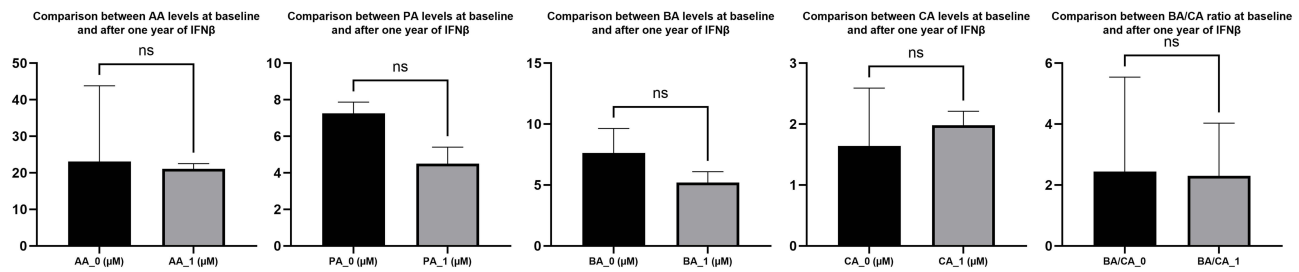
## Assessment of AA, PA, BA and CA Levels Based on Clinical and Demographical Characteristics at Baseline

No statistically significant differences were found between the levels of AA, PA, BA and CA and socio-clinical characteristics at baseline. Data is summarized in Table 5.

**Table 3** Median SCFA Values at Baseline and After One Year of IFN $\beta$  Treatment

	AA ( $\mu\text{M}$ )		PA ( $\mu\text{M}$ )		BA ( $\mu\text{M}$ )		CA ( $\mu\text{M}$ )		BA/CA ratio	
	Median	95% CI	Median	95% CI	Median	95% CI	Median	95% CI	Median	95% CI
<b>Baseline</b>	23.12	[15.84; 43.82]	7.250	[4.32; 7.87]	7.63	[2.70; 9.64]	1.64	[1.47; 2.59]	2.44	[1.02; 5.54]
<b>After one year of IFN<math>\beta</math> treatment</b>	21.10	[15.71; 22.53]	4.500	[3.88; 5.40]	5.19	[3.19; 6.09]	1.98	[1.37; 2.21]	2.3	[1.27; 4.03]
<i>p-value</i>	0.11		0.06		0.15		0.41		0.84	

**Abbreviations:** AA, acetic acid; PA, propionic acid; BA, butyric acid; CA, caproic acid, IFN $\beta$ , interferon beta. Wilcoxon matched-pairs signed rank test.



**Figure 1** Boxplot type diagrams with median and 95% CI showing comparison analysis of baseline SCFAs/MCFA levels and after one year of IFN $\beta$  treatment (all  $p > 0.05$ ). **Abbreviations:** AA, acetic acid; PA, propionic acid; BA, butyric acid; CA: caproic acid).

## Correlations Between Baseline AA, PA, BA and CA Levels and Studied Biomarkers in nMS

To assess whether the levels of SCFAs/MCFA were associated with the proposed biomarkers, we performed a correlation analysis. The analysis revealed a strong positive correlation between BA and PA ( $r = 0.70$ ,  $p = 0.0002$ ) and negative correlations between AA and MMP9 ( $r = -0.55$ ,  $p = 0.006$ ), CA and IL-10 ( $r = -0.47$ ,  $p = 0.02$ ), MMP9 and IL-10 ( $r = -0.56$ ,  $p = 0.005$ ). No statistically significant correlations were identified between the levels of SCFAs/MCFA and the clinical parameters. Graphical interpretations and Spearman's heatmap are summarized in Figures 3 and 4.

No statistically significant correlations were noted between the SCFAs/MCFA levels and the clinical characteristics of the patients.

Linear regression analysis was conducted to explore the relationship between SCFA/MCFA levels and inflammatory biomarkers. A statistically significant positive association was found between PA and BA levels, with approximately 40% of the variance in BA levels being explained by PA. By assessing the relationship between CA and IL-10 we found that IL-10 explained only a minimal proportion of the variance in CA levels without reaching statistical significance. The analysis of the relationship between AA and MMP9 demonstrated that MMP9 levels significantly predicted AA levels, explaining a moderate proportion of the variance in AA levels. Moreover, linear regression analysis conducted to examine the relationship between MMP9 and IL-10 levels explained a moderate proportion of the variance in MMP9 levels. The data is summarized in Table 6.

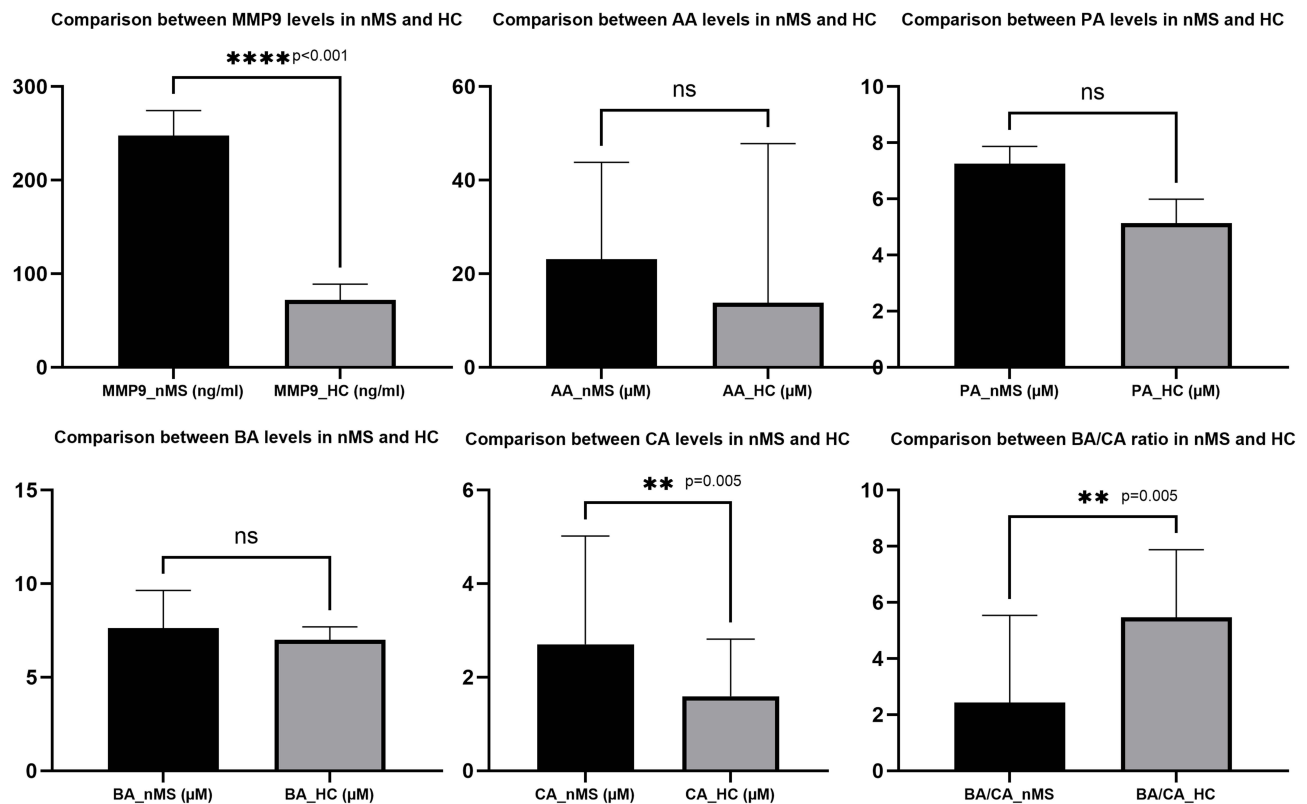
## Discussion

IFN $\beta$  was the first approved DMT for MS and remains a practical treatment option for RRMS due to its proven efficacy in reducing relapse rates, delaying disability progression, and reducing T2 lesion burden, with patients being excellent responders in approximately 30% of the cases.<sup>27</sup> The effects of DMTs, such as IFN $\beta$  are still under debate, depending on whether the anti-inflammatory effects result from systemic immunomodulatory effects that shift the immune responses

**Table 4** Comparison Between nMS and HC MMP9 and SCFA Values (Unpaired T-Test with Welch's Correction and Mann Whitney U. Data is Expressed as Mean  $\pm$  SD or as Median (95% CI))

	nMS (n=23)	HC (n=23)	p-Value
<b>MMP9 (ng/mL)</b> <sup>o</sup>	252.4 $\pm$ 87.53	72.03 $\pm$ 26.32	<0.001
<b>AA (<math>\mu</math>M)</b> <sup>¥</sup>	23.12 [15.84; 43.82]	13.79 [10.88; 47.82]	0.27
<b>PA (<math>\mu</math>M)</b> <sup>¥</sup>	7.250 [4.32; 7.87]	5.130 [4.61; 5.99]	0.14
<b>BA (<math>\mu</math>M)</b> <sup>¥</sup>	7.63 [2.70; 9.64]	7.010 [6.66; 7.69]	0.99
<b>CA (<math>\mu</math>M)</b> <sup>¥</sup>	1.64 [1.47; 2.59]	1.27 [1.04; 1.48]	0.005
<b>BA/CA</b> <sup>¥</sup> ratio	2.44 [1.02; 5.54]	5.47 [4.35; 7.88]	0.005

**Abbreviations:** AA, acetic acid; BA, butyric acid; CA, caproic acid; HC, healthy controls; IL, interleukin; MMP, matrix metalloproteinase; nMS, naïve MS; PA, propionic acid <sup>o</sup>, parametric distribution based on Shapiro–Wilk test of normality; <sup>¥</sup>, non-parametric distribution based on Shapiro test of normality;



**Figure 2** Boxplot type diagrams with median and 95% CI. Comparisons between MMP9, AA, PA, BA and CA between nMS and HC (\*\*\*\* extremely high significance, \*\* moderate significance).

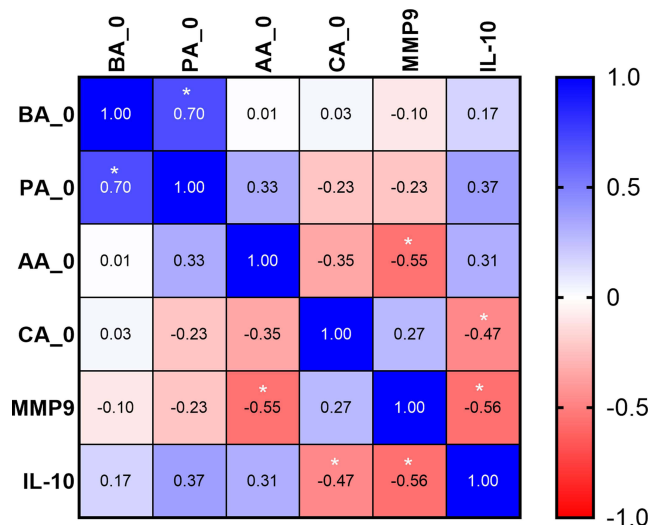
**Abbreviations:** AA, acetic acid; BA, butyric acid; CA, caproic acid; HC, healthy controls; PA, propionic acid; MMP9, matrix metalloproteinase; nMS, naive multiple sclerosis.

towards a Th2, or they carry direct interactions with the gut microbiome.<sup>24</sup> It was demonstrated that IFNβ has direct effects on commensal flora and communicates with the gut epithelial cells by regulating the homeostatic IFN-I response. IFN-I is involved in the regulation of immune responses in response to viral pathogens.<sup>28</sup> *Bacteroides fragilis*, a gram-negative anaerobic bacterium that resides in the colon, triggers the IFNβ expression by intestinal dendritic cells through signaling via Toll-like receptor 4, enhancing host resistance to viral infections.<sup>29</sup> Depending on the enterotoxin producing status, non-enterotoxin-producing *Bacteroides fragilis* is considered a next-generation probiotic with anti-inflammatory properties, by increasing levels of SCFAs, particularly AA and BA.<sup>30</sup> Additionally, basal IFNβ regulates the expression of interferon-stimulated genes.<sup>31</sup> Therefore, it is possible that this specific induction of IFNβ by *Bacteroides* strains contributes to homeostasis.

**Table 5** SCFA Value Comparison Across Gender, Living Environment and Disability. Data is Expressed as Median (95% CI); Unpaired Mann Whitney U

	Gender			Living Environment			Disability		
	Female (n=14)	Male (n=9)	p	Rural (n=10)	Urban (n=13)	p	EDSS_0 ≤ 1.0 (n=11)	EDSS_0 > 1 (n=12)	p
BA (μM) *	5.11 [1.19; 9.99]	8.48 [3.96; 10.05]	0.15	7.57 [1.19; 9.87]	8.41 [2.53; 10.41]	0.41	8.41 [1.19; 11.0]	7.61 [2.70; 9.99]	0.97
PA (μM) *	6.31 [3.80; 10.69]	7.25 [5.07; 7.94]	0.92	7.26 [3.64; 7.87]	5.62 [4.32; 10.69]	0.28	7.31 [3.94; 8.76]	6.16 [3.96; 7.94]	0.44
AA (μM) *	24.37 [11.56; 73.82]	23.12 [9.65; 112.7]	0.78	21.95 [9.89; 31.25]	38.06 [13.19; 149.0]	0.28	31.25 [9.65; 149.0]	18.68 [13.19; 38.06]	0.48
CA (μM) *	1.86 [1.38; 2.77]	1.59 [1.39; 4.39]	0.97	1.56 [1.38; 4.04]	1.86 [1.47; 4.39]	0.60	1.49 [1.39; 2.77]	1.90 [1.38; 4.39]	0.41
BA/CA ratio *	1.76 [0.59; 5.61]	4.92 [1.52; 6.58]	0.20	3.09 [0.59; 6.65]	2.44 [1.02; 5.61]	0.76	2.44 [0.59; 6.65]	2.44 [0.98; 5.54]	0.78

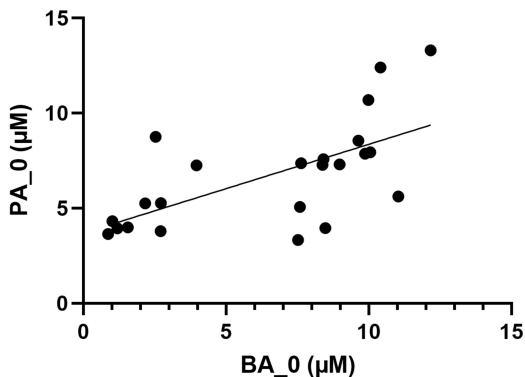
**Abbreviations:** AA, acetic acid; BA, butyric acid; CA, caproic acid; EDSS\_0, Expanded Disability Status Scale; PA, propionic acid; \* non-parametric distribution based on Shapiro test of normality.



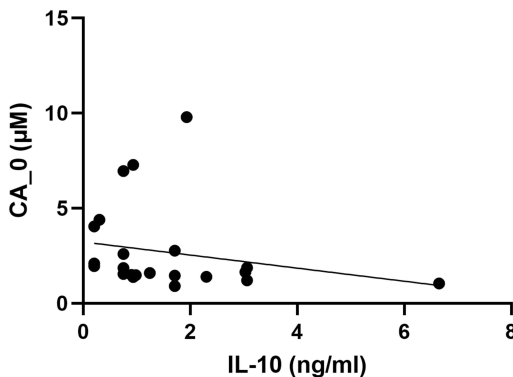
**Figure 3** Heatmap displaying correlations between SCFA, MMP9, and IL-10. Each cell represents the correlation coefficient with colors indicating the strength and direction of correlation. The statistically significant correlations are marked with \* (Spearman's rho).

**Abbreviations:** AA, acetic acid; BA, butyric acid; CA, caproic acid; IL, interleukin; MMP, matrix metalloproteinase; PA, propionic acid.

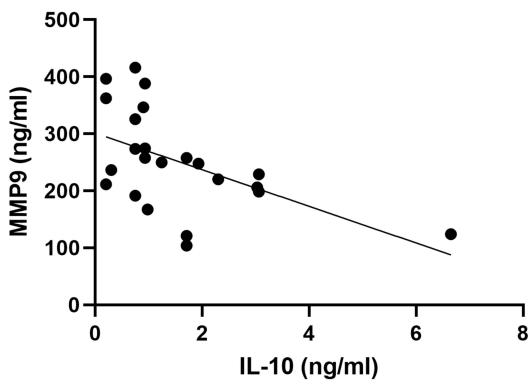
**Correlation analysis between PA\_0 and BA\_0**  
 $r=0.70, p<0.001$



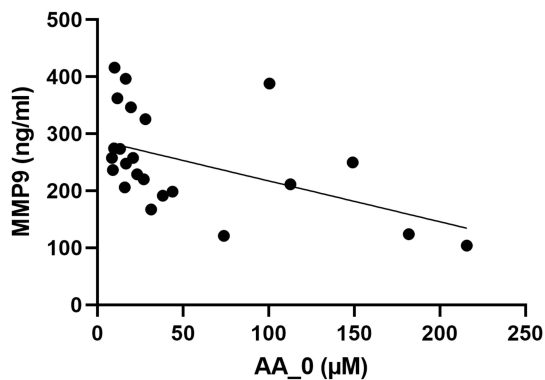
**Correlation analysis between CA\_0 and IL-10**  
 $r=-0.47, p=0.02$



**Correlation analysis between MMP9 and IL-10**  
 $r=-0.56, p=0.005$



**Correlation analysis between MMP9 and AA\_0**  
 $r=-0.55, p=0.006$



**Figure 4** Correlations between PA and BA, CA and IL-10, MMP9 and IL-10, MMP9 and AA (Scatter Plot, Spearman's Rho).

**Abbreviations:** AA, acetic acid; BA, butyric acid; CA, caproic acid; IL, interleukin; MMP, matrix metalloproteinase; PA, propionic acid.

**Table 6** Linear Regression Results

Biomarker	Predictor	$\beta$	R <sup>2</sup>	Adjusted R <sup>2</sup>	SE	95% CI	p
BA	PA	0.87	0.40	0.38	0.23	[0.39; 1.35]	0.001
CA	IL-10	-0.34	0.04	0.00	0.34	[-0.10; 0.37]	0.32
AA	MMP9	-0.33	0.23	0.20	0.13	[-0.60; -0.64]	0.01
MMP9	IL-19	-0.32	0.27	0.24	76.2	[-0.55.71; -9.50]	0.01

**Abbreviations:** AA, acetic acid; BA, butyric acid;  $\beta$ , estimated coefficient; CA, caproic acid; IL, interleukin, MMP, matrix metalloproteinase; SE, standard error; R<sup>2</sup>, coefficient of determination.

As of now, no longitudinal studies have been conducted on the assessment of peripheral SCFAs/MCFA in treatment-naïve vs DMT treated MS patients. The study population consisted of nMS patients and HC with similar characteristics in terms of socio-demographical characteristics. The patients were selected to be recently diagnosed, with a disease evolution of less than 1 year until the initiation of IFN $\beta$  therapy. In our study, while there was a slight trend towards decreased SCFA levels and increased CA levels after one year of IFN $\beta$  treatment, these changes were not statistically significant and baseline SCFAs/MCFA levels did not differ from the ones of HC.

The microbiota-centered studies involving PwMS treated with IFN $\beta$  involve a direct assessment of bacterial phyla through stool samples. Castillo-Álvarez et al evaluated a cohort of PwMS, comparing those treated with IFN $\beta$  against untreated PwMS and HC, based on the bacterial phyla from stool samples. They demonstrated that PwMS, whether treated or untreated, exhibited alterations in the abundance of *Firmicutes* and *Actinobacteria*, compared to HC.<sup>32–34</sup> These phyla represent the main BA producers in the human microbiome and the results showed significant differences between HC and untreated PwMS but not between HC and treated PwMS. This could indicate that IFN $\beta$  therapy may have a stabilizing effect on the gut microbiota, bringing it closer to the composition observed in HC.<sup>32–34</sup> Similar results were reported by Jangi et al in cohorts treated with IFN $\beta$  and glatiramer acetate. While no significant differences were noted between the two DMTs, indicating that the observed effects may not be specific to IFN $\beta$  treatment but rather to immunomodulatory therapy in general, differences were noted in relation to different bacterial phyla. For instance, *Prevotella*, an important SCFA producer, was increased in treated PwMS compared to untreated PwMS.<sup>35</sup> Zhou et al described an increase in PA in serum samples in IFN $\beta$  treated patients while the same effect was not reported in the other treatment groups.<sup>36</sup>

We found no differences in serum levels for the analyzed SCFA – AA, PA and BA between nMS and HC. BA and PA share metabolic pathways and cross-feeding interactions. Some bacteria abundant in the human gastrointestinal system, such as *Faecalibacterium prausnitzii*, are main BA synthesizers but can also produce PA under certain conditions. *Eubacterium hallii* can generate both BA and PA from amino acids and complex carbohydrates. This can explain the strong positive correlation we found between these two SCFAs, and this finding warrants future studies comparing peripheral SCFA concentrations with the bacterial taxonomy of BA and PA producers.<sup>37,38</sup>

The levels of CA were found to be higher in nMS patients compared to HC. While there were no differences in BA, PA, and AA levels, the proposed ratio of BA/CA was significantly higher in HC compared to nMS. While some fecal SCFA-centered studies reported lower levels of BA in PwMS compared to HC,<sup>39–42</sup> in our group, there were no statistically significant differences. This can be partly explained by the fact that the patients have recently been diagnosed with MS and the intestinal dysbiosis is in the early stages, difficult to quantify in the periphery. In MS, BA has important immunomodulatory properties, ranging from the activation of GPRs which, in turn, stimulate the secretion of IL-10 with anti-inflammatory properties, to HDAC-I that drive Treg cell differentiation, modulating the inflammatory immune responses towards protective rather than encephalitogenic.<sup>12,43–45</sup> CA is an MCFA that favors Th1 and Th17 differentiation, thus having pro-inflammatory effects. MCFAs control carbohydrate and lipid metabolism and play important roles in mitochondrial energy<sup>15</sup> but it is suggested to also have destructive implications in MS. The ratio between BA/CA concentrations reflects the Treg/Th1 axis balance which is typically disrupted in MS, as proposed by Sarasella et al.<sup>40</sup> The relationship between CA and IL-10 revealed that IL-10 explained only a minimal proportion of the differences in CA levels and did not reach statistical significance. This suggests that CA levels is not influenced by IL-10 serum concentrations in the studied population.

MMP9, a member of the MMP family is an enzyme involved in the breakdown of extracellular matrix components with attested roles in intestinal barrier and BBB permeability.<sup>21,46</sup> In nMS patients, IL-10 is negatively correlated with MMP9 activity and this effect was demonstrated to be dependent of the IL-10 levels.<sup>47</sup> A reduced level of IL-10 has been associated with an increased risk of developing MS and greater disease aggressiveness. IL-10 acts as an anti-inflammatory cytokine that modulates immune responses and promotes neuroprotection by attenuating inflammatory processes and facilitating tissue repair.<sup>48,49</sup> Our results are congruent with the reported data, supporting the beneficial effect of this anti-inflammatory cytokine in reducing inflammation in MS.

Additionally, we observed a significant negative correlation between MMP9 and AA levels. Although there are currently no reports regarding the role of AA in BBB maintenance, its levels are sensitive to inflammatory biomarkers. Pérez-Pérez et al demonstrated that AA levels were higher in patients with EDSS  $\geq$  5.0 compared to the ones with lesser disability<sup>50</sup> while Olsson et al reported that AA levels negatively correlated with other pro-inflammatory biomarkers, such as IFN- $\gamma$ .<sup>41</sup> Interestingly, in ulcerative colitis models, AA administration was associated with an increased protection of the intestinal barrier both in inflammatory and homeostatic conditions.<sup>51</sup> Our results suggest that higher AA levels could be considered as a possible protector of BBB permeability.

Across different genders, living environments and disability levels, there were no statistically significant differences in the serum levels of the studied metabolites. This suggests that factors such as sex, living environment and disability status do not significantly influence the concentrations of SCFA and MCFA in the study population. It's essential to consider that these findings may vary in different study populations and across MS phenotypes and further research is needed to confirm these results.

The strength of this study is represented by the homogenous study population (new onset of the disease, mild disease burden, same DMT regimen) and prospective collection of data regarding the peripheral metabolites of gut microbiota, SCFA/MCFA in nMS before and after one year of IFN $\beta$  treatment, along with the high-performance validated method for SCFA/MCFA assessment using state-of-the-art highly selective, sensitive, accurate and precise time-of-flight mass spectrometric detection and ultra-high performance liquid chromatographic separation. The study limitations include a relatively small sample size, but these are patients that clinically fit the profile for IFN $\beta$  treatment and the patient number does not differ greatly from other studies reporting on the subject; the great variability of the gut microbiota and the lack of known dietary regime. Due to the financial aspect, we were not able to evaluate the taxonomic profiles of gut microbiome by gene sequencing that would have mirrored the SCFA/MCFA activity; this would constitute the subject of further studies. Our findings contribute to the understanding of gut microbiota in nMS patients and the potential of peripheral SCFA/MCFA as prognostic biomarkers.

## Conclusions

In summary, in our cohort, we have demonstrated that IFN $\beta$  does not modify the peripheral SCFA/MCFA levels in nMS patients at baseline compared to one year after the initiation of therapy. Further studies on larger cohorts including different phenotypes of MS (RRMS, SPMS) are required. Higher levels of CA were found in nMS patients and conversely, a lower ratio of BA/CA. SCFA/MCFA levels are regulated by inflammatory biomarkers such as MMP9 and IL-10 and further studies on larger MS cohorts are necessary in order to expand this observation.

## Abbreviations

AA, acetic acid; BA, butyric acid; BBB, blood-brain barrier; BMI, body mass index; CA, caproic acid; CNS, central nervous system; DMT, disease-modifying therapy; EDSS, Expanded Disability Status Score; GPCR, G protein-coupled receptors; HC, healthy controls; HDAC-i, histone deacetylase activity inhibitor; IFN $\beta$ , interferon beta; IL, interleukin; MCFA, medium-chain fatty acids; MMP, matrix metalloproteinases; MS, multiple sclerosis; nMS, naïve MS patients; PwMS, patients with multiple sclerosis; PA, propionic acid; RR, relapsing-remitting; SCFA, short-chain fatty acids; Treg, regulatory T cells; Teff, effector T cells; Th, T helper.

## Data Sharing Statement

Data are contained within the article and is available upon reasonable request. Please Email the first author at laurabarcucean@gmail.com.

## Institutional Review Board Statement

The study was conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee of the Emergency Clinical County Hospital of Targu Mures (Approval No. 13555/21.06.2022) and the Ethics Committee of the University of Medicine, Pharmacy, Science and Technology “George Emil Palade” of Targu Mures (Approval No. 1832/14.07.2022).

## Informed Consent Statement

Informed consent was obtained from all subjects involved in the study.

## Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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## Disclosure

The authors declare no conflicts of interest.

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