Urine Metabolite Analysis to Identify Pathomechanisms of Long COVID: A Pilot Study

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ABSTRACT

BACKGROUND: Around 10% of people who had COVID-9 infection suffer from persistent symptoms such as fatigue, dyspnoea, chest pain, arthralgia/myalgia, sleep disturbances, cognitive dysfunction and impairment of mental health. Different underlying pathomechanisms appear to be involved, in particular inflammation, alterations in amino acid metabolism, autonomic dysfunction and gut dysbiosis.

AIM: As routine tests are often inconspicuous in patients with Long COVID (LC), similarly to patients suffering from myalgic encephalomyelitis/chronic fatigue syndrome (ME/CFS), accessible biomarkers indicating dysregulation of specific pathways are urgently needed to identify underlying pathomechanisms and enable personalized medicine treatment. Within this pilot study we aimed to proof traceability of altered metabolism by urine analysis.

PATIENTS AND METHODS: Urine metabolome analyses were performed to investigate the metabolic signature of patients with LC (n = 25; 20 women, 5 men) in comparison to healthy controls (Ctrl, n = 8; 7 women, 1 man) and individuals with ME/CFS (n = 8; 2 women, 6 men). Concentrations of neurotransmitter precursors tryptophan, phenylalanine and their downstream metabolites, as well as their association with symptoms (fatigue, anxiety and depression) in the patients were examined.

RESULTS AND CONCLUSION: Phenylalanine levels were significantly lower in both the LC and ME/CFS patient groups when compared to the Ctrl group. In many LC patients, the concentrations of downstream metabolites of tryptophan and tyrosine, such as serotonin, dopamine and catecholamines, deviated from the reference ranges. Several symptoms (sleep disturbance, pain or autonomic dysfunction) were associated with certain metabolites. Patients experiencing fatigue had lower levels of kynurenine, phenylalanine and a reduced kynurenine to tryptophan ratio (Kyn/Trp). Lower concentrations of gamma-aminobutyric acid (GABA) and higher activity of kynurenine 3-monooxygenase (KMO) were observed in patients with anxiety. Conclusively, our results suggest that amino acid metabolism and neurotransmitter synthesis is disturbed in patients with LC and ME/CFS. The identified metabolites and their associated dysregulations could serve as potential biomarkers for elucidating underlying pathomechanisms thus enabling personalized treatment strategies for these patient populations.

KEYWORDS: Long-COVID, fatigue, urine metabolites, gut dysbiosis, IFN-gamma mediated pathway

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Introduction

Persistent symptoms after COVID-19 still pose a huge challenge for the health care system.[1-3] Most affected individuals who do not recover fully within 4 to 12 weeks, suffer from persistent fatigue,[1,2] but also other symptoms like memory and concentration deficits, sleep disturbances, pain, dyspnoea, orthostatic intolerance or depressive symptoms are regularly reported by 'Long haulers'. Among these symptoms, post-infectious fatigue is often the most disabling symptom for patients.[2] Post-infectious fatigue can also occur after other infections (like Epstein Barr virus or herpes virus infections)[4,5] and goes along with a significant loss of physical and/or mental functionality that can be worsened by post-exertional malaise (i.e, the worsening of fatigue or other symptoms after physical, emotional or mental over-exertion). Sleep is often described as not restful by patients,[6,7] and also other symptoms like cognitive impairment or autonomic dysfunction can reduce the patients' ability to work significantly. Some patients are not even capable to care for themselves anymore, because they develop myalgic encephalitis (ME/CFS).[8] ME/CFS is a systemic multi-organ disease, which impairs the patients' ability to work and their quality of life significantly. It has long been misdiagnosed as psychiatric disease, as there are no biomarkers and symptom presentation varies greatly between patients.[9,10] The aetiology of ME/CFS as well as LC often remains unclear, actually many different pathomechanisms have been described.[4-6,11-17]

Numerous studies on ME/CFS have previously noted alterations in the intestinal microbiota and dysbiosis,[18-20] although

the available data remains inconclusive.[21] As of now, a specific and consistently recurring microbial pattern has not been established.[20,22] Given the similarities between ME/CFS and LC, it has been suggested that changes in gut microbiota may also play a role in the development of long COVID conditions.[23] Gastrointestinal issues persisting 7 months after the initial SARS-CoV-2 infection are notably more common among individuals who had a moderate-to-severe form of acute COVID-19 compared to asymptomatic patients or those with a mild presentation of the disease. The primary enduring gastrointestinal symptoms post-COVID are diarrhoea and abdominal pain.[24] Moreover, gut dysbiosis can lead to inflammatory processes through the release of lipopolysaccharides into the circulation, which may also negatively impact brain function[25] and lead to the enhanced formation of pro-inflammatory cytokines.[26]

Recently, pro-inflammatory cytokines have been associated with the development of behavioural symptoms[27-29] in patients with LC as well as in patients with ME/CFS.[29,30] Also immune activation and immune-mediated changes of tryptophan metabolism in the blood have been associated with fatigue after EBV-infection[5] and fatigue in patients with cancer.[30] Similarly, depression and impaired quality of life were associated with immune-mediated alterations of serum tryptophan catabolism in patients suffering from HIV-infection[31] and cancer.[32] In male patients with solid tumours enhanced tryptophan catabolism coincided with depression and anaemia, while phenylalanine accumulation was associated with a decreased quality of life in female patients.[33] Increased blood phenylalanine concentrations are also related with lower quality of life and lower mental and social skills in patients with phenylketonuria,[34] an inborn error of metabolism, which can only be treated by a special low protein diet.

Phenylalanine and tryptophan levels are thus not only influenced by immune activation, but also by diet, gut function and microbiota. For instance, impaired gut function, as seen in conditions like Crohn's disease accompanied by damage to gut epithelial cells and tight junctions, can reduce the absorption of essential nutrients, including of amino acids. Furthermore, a 'leaky' gut barrier can also lead to translocation of for example, lipopolysaccharides or other bacterial toxins to the blood circulation and thereby contribute importantly to systemic inflammatory processes.[25,35-37] The composition and diversity of the gut microbiota play a crucial role in maintaining gut barrier integrity, and a decrease in diversity has been linked to neurodegenerative diseases.[38] Considering the significance of the gut-brain axis, dysbiosis of the gut microbiota is proposed as a contributing factor to post-infectious fatigue, further highlighting its impact on overall well-being.[39]

Neuroinflammatory processes consecutive to gut dysbiosis in fact appear to be a main underlying pathomechanism of cognitive decline, fatigue and depression, and are probably also involved in patients with LC and ME/CFS.[25,40-42] A leaky gut barrier with reduced absorption/metabolization of nutrients by commensal gut microbiota might also explain other metabolic consequences, which need to be studied comprehensively: Downstream catabolites of the tryptophan and phenylalanine pathway are necessary for neurotransmitter synthesis (eg, serotonin, dopamine or catecholamines), and mitochondrial function (eg, nicotinamide adenine dinucleotide [NAD]). In fact many symptoms of patients (like fatigue, sleep disturbances or feeling depressed) have been associated with immune-mediated alterations of tryptophan metabolism and decreased B vitamin availability.[32,43]

As dysregulation of neurotransmitter formation and mitochondrial dysfunction might explain fatigue, dysautonomia, sleep or mood disturbances of patients with LC and ME/CFS, we aimed to investigate the above mentioned biochemical pathways involved in neurotransmitter formation in both patient groups. In this proof of concept study concentrations of metabolites of the tryptophan and phenylalanine pathways, and neurotransmitters in the urine and their correlation with symptoms were investigated.

Materials and Methods

Study population

Acid stabilized urines of 32 subjects with LC, 12 healthy controls (Ctrl) and 12 subjects with ME/CFS were collected for urine metabolite analyses between June and December 2022 at the Department of Internal Medicine II of the University Hospital Innsbruck, Austria.

Patients included into the LC cohort were classified according to the CDC classification. They had suffered from symptoms in terms of a Long COVID condition for longer than 6 months. With a few exceptions (4 patients), the symptoms had already existed for longer than 1 year-the median duration of symptoms was 567 days.

For statistical analyses, only the results from patients with urine creatinine levels within the reference range were included. This was done to ensure that the concentrations of other parameters were not affected by dilution or excessive concentration. The final study population consisted of 25 patients suffering from LC, 8 patients with diagnosed ME/CFS and 8 healthy controls (Ctrl). The median age was similar in all 4 groups (LC: 38; ME/CFS: 36.25; Ctrl: 28). In total more women (n = 29) were recruited within the study (LC: 20 female, 5 male; ME/CFS: 2 female, 6 male; and Ctrl: 7 female, 1 male).

Acid stabilized second morning urines were collected (2-4 hours after the first morning urine) according to the instructions provided by Biovis (Limburg, Germany). No fish was allowed to be eaten 2 days before the urine was collected, as well as, no chocolate, nuts or supplements containing omega 3 fatty acids in the evening before the examination. Patients with LC condition and ME/CFS, who had presented with ongoing symptoms after COVID-19 or other infections (mostly

EBV-infection), and who gave written informed consent were included into the study. The study conformed to the ethical principles outlined in the Declaration of Helsinki and was approved by the ethical committee of Innsbruck Medical University to store urine and blood specimens for further analyses in a biobank (EK-Nr 2017/1157) and to answer questionnaires in the scope of the PRECISE study (EK-Nr 1103/2020), addressing fatigue, anxiety, depression, trauma and somatic symptoms as detailed below.

Canadian consensus criteria

The Canadian Consensus Criteria questionnaire was administered to evaluate whether subjects exhibited symptoms consistent with Myalgic Encephalomyelitis/Chronic Fatigue Syndrome (ME/CFS). Subjects were asked about: (1) exhaustion/fatigue and worsening of condition after exertion; (2) sleep disorders; (3) pain; (4) neurological/cognitive manifestation; (5) autonomic nervous system manifestation; (6) neuroendocrine manifestation; (7) immunological manifestations. For a diagnosis of ME/CFS, criteria (1) must all be met, at least one for (2) sleep disturbances and (3) pain; 2 or more for (4) neurological/cognitive manifestations and one or more symptoms of at least 2 of the categories (5) autonomic, (6) neuroendocrine and (7) immune manifestations. Patients who had autonomic manifestations presented with one or more of the following symptoms: orthostatic hypotonia/drowsiness, dizziness, postural tachycardia, extreme paleness, intestinal disorders [diffuse abdominal pain, bloating, burning sensations], bladder disorders, palpitations, dyspnoea with light exertion.

DePaul Symptom-Subscale Questionnaire

The DePaul Symptom Questionnaire was utilized to evaluate the presence of myalgic encephalomyelitis (ME) and chronic fatigue syndrome (CFS) symptoms in the subjects. The questionnaire determines the severity and frequency of ME/CFS symptomatology, and the exertion intolerance. Study participants rated the frequency of their symptoms and their severity over the last 6 months in a 5-point Likert scale. The rating scale included the following levels: 0 (none of the time/symptom not present), 1 (mild intensity), 2 (moderate intensity), 3 (severe intensity) and 4 (very severe intensity). The exertion intolerance is scored depending on how long it takes after an activity to feel well again (0 hours up to 3 days).

A frequency of at least 2 and a severity of at least 2 on any of the 5 questions of the DSQ_PEM subscale indicates that PEM is present. A frequency of 2 on one question and a severity of 2 on another question does not meet the criteria for PEM. A duration of at least 14 hours is necessary for the diagnosis of ME/CFS.

PHQ-D Questionnaire

The PHQ-D questionnaire is a screening instrument, which in combination with the medical consultation enables a valid and time-efficient diagnosis of mental disorders. The complete version enables the diagnosis of somatoform disorders, depressive disorders, anxiety disorders, eating disorders and alcohol abuse. In this pilot study we focused on the subcategories depression and anxiety, which are analysed by 3 questions of the PHQ-9 questionnaire and GAD-7 score. The questions determine if the subjects of the study had a panic attack in the last 4 weeks and its severity and if the subjects felt nervousness, anxiety, tension or excessive apprehension in the last 4 weeks. Patients were considered to experience a panic syndrome if they responded affirmatively to each of the sub-questions in question 1, and 4 or more of the sub-questions in question 2. For other anxiety syndromes, if the subjects answered affirmatively to 4 or more sub-questions in question 3, specifically indicating occurrence 'on more than half of the days in the last 2 weeks', it suggested the presence of such syndromes.

SSS-8 Questionnaire

As a short, patient-reported outcome measure of somatic symptom burden, the 8-item Somatic Symptom Scale (SSS-8) was developed. In a 5-point Likert scale, respondents rate their perceived level of discomfort with common somatic symptoms within the last 7 days. An average is calculated based on the sum of the ratings. Scores range from 0 to 32. The severity categories are as follows: 0 to 3 None to minimal; 4 to 7 low; 8 to 11 medium; 12 to 15 high and 16 to 32 very high. The following symptoms are included in the questionnaire: stomach or bowel problems; back pain; pain in your arms, legs or joints; headaches; chest pain or shortness of breath; dizziness; feeling tired or having low energy; trouble sleeping.[44]

SSD-12

In somatic stress disorders, the SSD-12 questionnaire is used to record psychological stress. Twelve items are included in the SSD-12. Psychological sub criteria for somatic symptom disorders are measured through 4 items each (cognitive, affective and behavioural). Points were assigned based on the responses provided by the subjects, with the following scale: 0 (never), 1 (rarely), 2 (sometimes), 3 (often) and 4 (very often). A simple sum score is calculated based on the ratings (between 0 and 48 points). A sum of over than 23 points indicates a risk for SSD.[45]

The Primary Care PTSD Screen for DSM-5 trauma evaluation

The Primary Care PTSD Screen for DSM-5 (PC-PTSD-5) is a 5-item screening tool, which was developed to screen primary care patients for probable PTSD. Initially, the measure

examines lifetime exposure to traumatic events. Respondents who deny exposure receive a score of 0 on the PC-PTSD-5. Subjects who have been exposed to trauma over the past year are required to answer 5 additional 'yes/no' questions about how that trauma has affected them. The PC-PTSD-5 allows respondents to score a 0 to 5 based on the number of affirmative responses.

Laboratory measurements

Metabolome analyses of the acid stabilized second morning urine samples were performed by Biovis Diagnostik MVZ GmbH: concentrations of tryptophan and phenylalanine metabolism with all important downstream metabolites (kynurenine, kynurenic acid, 3-OH-kynurenine, quinolinic acid), cofactors (nicotinic acid, nicotinamide adenine dinucleotide, cystathionine) catecholamines (dopamine, noradrenaline and adrenaline) and neurotransmitters (glutamate, gamma-aminobutyric acid [GABA], serotonin), as well as chronic inflammation (as reflected by T-helper cells type 1 immune activation marker neopterin) were measured. Enzyme activities (kynurenine to tryptophan ratio, phenylalanine to tyrosine ratio, kynurenine-3-monooxygenase) were calculated.

The preparation of the urine samples involved combining 25 µl of stabilized second morning urine (or urine calibrator, quality control) with 250 µl of solvent A and 20 µl of the internal standard mix. Following vortexing and centrifugation, 6 µl of the resulting supernatant was injected into the LC/MS-MS system. 50 µl of 20% hydrochloric acid was dried on DBS cards and mixed with 10 ml of the second morning urine. The pH value was therefore <4 for all second morning urine samples.

Tryptophan metabolites from urine were analysed on a Triple-Quas MS 5500+ from sciex. Liquid chromatography (LC) separation was performed on an Agilent 1290 Infinity II with a Restek ARC-18 separation column (Stationary Phase C18, octadecylsilane (L1) 1.8 µm, 100 × 2.1 mm). The Raptor ARC-18 column uses these solvents: A-LC/MS grade water with 0.1% formic acid; B-LC/MS grade methanol with 0.1% formic acid and 0.01% trifluoracetic acid. The gradient at a constant flow of 0.37 ml/min was set as shown in Table 1.

Mass spectrometry (MS) settings in in positive electro spray ionization mode (ESI+) (tryptophan, kynurenine, 3-OH-kynurenine, kynurenic acid, neopterin, niacin, nicotinamide, nicotinamide adenine dinucleotide, tyrosine, phenylalanine) were curtain gas (CUR) 35.0; collision gas (CAD) 8; ion source voltage (IS) 5500; temperature (TEM) 500; gas 1 (GS1) 62; gas 2 (GS2) 60. MS settings in negative electro spray ionization mode (ESI-) (quinolonic acid) were CUR 35.0; CAD 8; IS −5500; TEM 500; GS1 62; GS2 60. Analyses were performed in multiple reaction monitoring mode with a separate isotopically labelled internal standard per analyte (except nicotinamide adenine dinucleotide).

Reference ranges of the analysed metabolites were determined internally based on the percentile distribution of n > 100 participants (healthy according to self-report) and are now continuously checked and optimized using statistical methods from meanwhile >10 000 samples. The method for determination of the above-mentioned metabolites was developed by Dipl. Chem. Till Heine of Biovis Diagnostik.

Table 1. Gradient settings of metabolome analysis.

TIME (MIN)	A (%)	B (%)	MAX. PRESSURE LIMIT (BAR)
0.00	97	3	1300
0.50	97	3	1300
0.95	70	30	1300
1.90	40	60	1300
2.40	40	60	1300
2.41	20	80	1300
4.10	20	80	1300
4.11	97	3	1300
5.70	97	3	1300

Statistical analysis

Statistical analyses were conducted using IBM SPSS Statistics Version 29 (IBM Corporation, Armonk, New York), while figures were generated using GraphPad Prism 9. The normal distribution of the data was assessed using the Kolmogorov-Smirnov test, which indicated non-normal distribution. Consequently, non-parametric tests including Mann-Whitney *U* and Kruskal-Wallis tests were employed. In cases where data were missing, they were treated as missing values. Statistical significance was defined as *P*-values <.05. Scatter dot plots with bars were used to depict the graphical representation of the data. Each symbol on the plot represents a measurement from an individual within the study population. The data are presented as means ± SEM. Heat maps were generated in R using the normalized data after applying the Z-score transformation to each metabolite. The Z-score normalization was performed by subtracting the mean value of each metabolite across all samples and dividing it by the standard deviation.

Results

Analysis of fatigue

In order to assess the symptoms exhibited by individuals, all participants, both patients and controls, were surveyed regarding the presence of symptoms typically employed for the classification of ME/CFS. These symptoms corresponded to the criteria outlined in the Canadian Consensus Criteria (CCC) for ME/CFS. A total symptom score was calculated by addition of all symptoms (one point per symptom). The 2 patient groups showed a significant symptom load, most significantly

pronounced in the ME/CFS group (Figure 1). Ctrl did not report about fatigue with PEM. Infection with COVID-19 in our study population went along with a slightly lower CCC score compared to patients with ME/CFS caused by other infections (*P*-value <.05).

Post-Exertional Malaise (PEM)-Scores were highest in patients with LC (mean ± SD = 29.18 ± 8.1), and lowest in healthy individuals (mean ± SD = 0.2 ± 0.4) (data not shown). In comparison to the Ctrl the LC cohort had significantly higher scores (*P*-value <.001), whereas the ME/CFS cohort (mean ± SD = 23.3 ± 16.5) had slightly lower scores than the LC cohort. Compared to the Ctrl, patients diagnosed with ME/CFS had significantly higher PEM scores (*P*-value <.02).

Notably, LC patients suffered more frequently and severely from fatigue compared to patients diagnosed with ME/CFS. However, the scores did not differ significantly between the 2 groups.

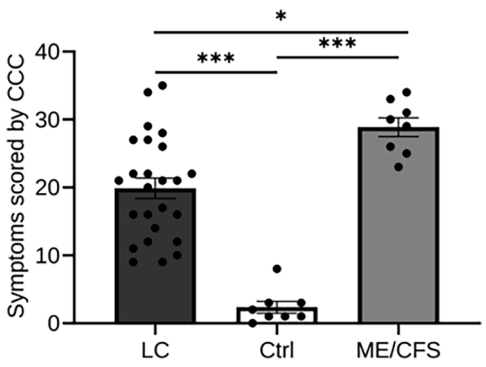

Figure 1. Symptom scores measured according to the Canadian Consensus Criteria (CCC) of participants; suffering from Long-COVID (LC; black column), healthy controls (Ctrl, white column) and subjects diagnosed with ME/CFS (grey column).
Significant differences were determined by Kruskal-Wallis with a Dunn post-hoc test.
**P*-value <.05. ***P*-value <.001.

Differences in stress, anxiety and somatic symptoms

As the symptoms reported for post-infectious fatigue were very diverse, we analysed somatic symptoms (SSS-8 questionnaire), stress (SSD-12 questionnaire), depression and anxiety (PHQ-D, GAD7 questionnaire) in the study cohort. The SSD-12 score, which measures psychological stress levels, was higher in LC and ME/CFS patients compared to the Ctrl. However, there was no significant difference observed between the 2 groups experiencing post-infectious fatigue (Figure 2A). Similarly, significantly higher levels of SSS-8, which detects somatic symptoms, were seen in the LC and ME/CFS cohort compared to CTRL. Subjects suffering from LC and subjects diagnosed with ME/CFS did not differ (Figure 2B).

Among the patients with LC, 10 individuals reported low psychosocial stress levels, 11 individuals reported medium levels, 2 individuals reported high levels and 2 individuals reported very high levels. The psychosocial stress level of Ctrl were low (except for one individual), while 5 patients with ME/CFS had medium and 2 had high psychosocial stress levels (one was low).

The data showed a marked difference between the groups, with LC patients demonstrating a much higher incidence of self-reported anxiety attacks (n = 13; 52%), while no such reports were found among the ME/CFS patients (n = 0). These results were also reflected by the PHQ-9 score and GAD7 score (Figure 3A). Patients suffering from LC had significantly higher depression scores compared to Ctrl, and tended to be more depressed than ME/CFS patients (Figure 3A). Similarly, anxiety levels tended to be higher compared to Ctrl and ME/CFS patients (Figure 3B).

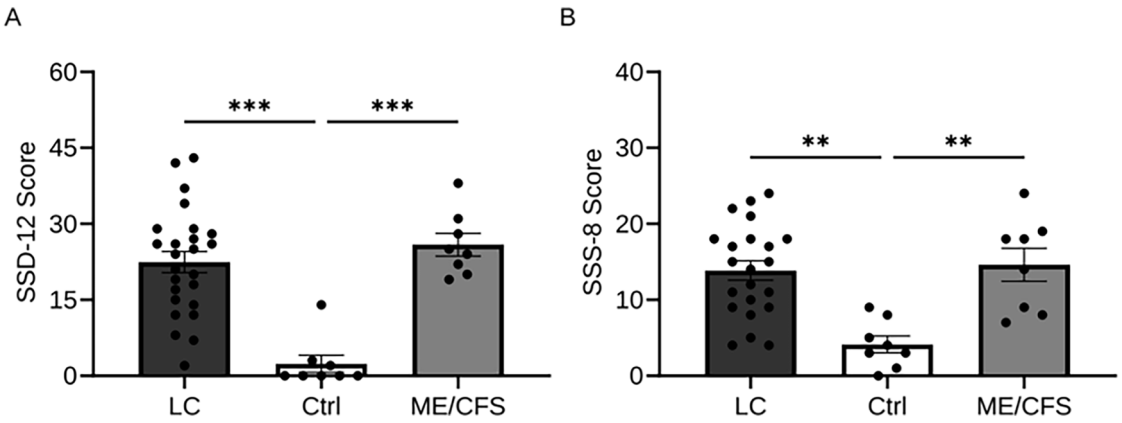

Figure 2. (A) SSD-12 score of participants suffering from Long-COVID (LC; black column), healthy controls (Ctrl, white column) and subjects diagnosed with ME/CFS (grey column). (B) SSS-8 score of participants divided into subjects suffering from LC (LC; black column), healthy controls (Ctrl, white column) and subjects diagnosed with ME/CFS (grey column).
Significant differences were determined by Kruskal-Wallis with a Dunn post-hoc test.
**P*-value <.01. ***P*-value <.001.

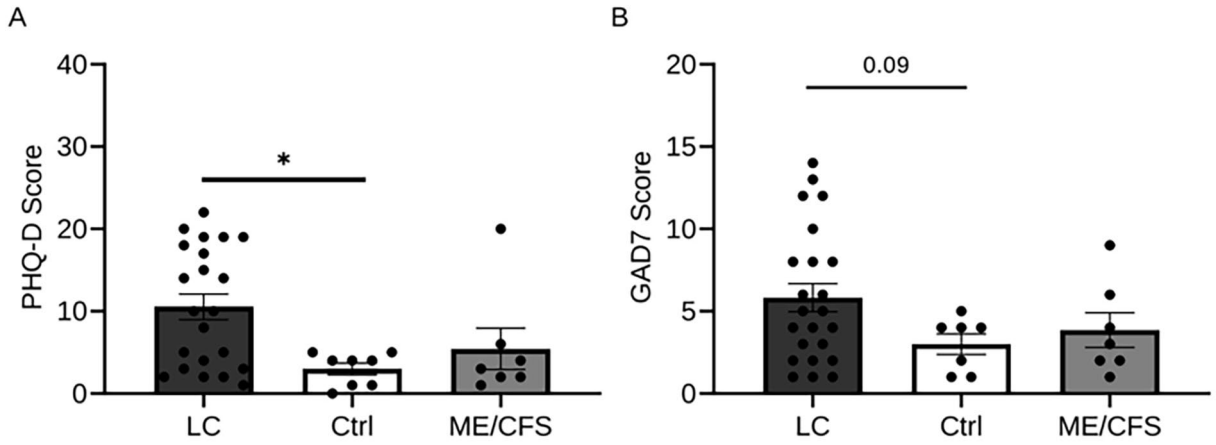

Figure 3. (A) PHQ-9 (depression) score of participants suffering from Long-COVID (LC; black column), healthy controls (Ctrl, white column) and subjects diagnosed with ME/CFS (grey column). (B) GAD7 (anxiety) score of participants divided into subjects suffering from Long-COVID (LC; black column), healthy controls (Ctrl, white column) and subjects diagnosed with ME/CFS (grey column).
Significant differences were determined by Kruskal-Wallis with a Dunn post-hoc test.
*P-value $<.05$.

Moreover, we analysed the results of the Primary Care PTSD Screen for DSM-5 (PC-PTSD-5). Nearly half of the patients with LC had scores indicating experience with trauma (n = 12; 48%), in patients with ME/CFS diagnosis 3 of 8 patients appeared to be traumatized (37.5%).

Dysregulation of metabolites analysed in urine of Long COVID patients

We determined concentrations of the most important IFN-γ-induced metabolic pathways metabolites in our study population. Tryptophan concentrations did not differ significantly between Ctrl, LC and ME/CFS (Figure 4A), but were rather lower in patients with post-infectious fatigue (see Figure 4). Patients with ME/CFS presented with lower levels of kynurenine, 3-OH-kynurenine, phenylalanine and the Kynurenine/Tryptophan ratio compared to Ctrl (Table 2, Figure 4B and D-F). Patients suffering from LC or ME/CFS showed significantly lower levels of phenylalanine compared to the Ctrl (Figure 4B).

Furthermore, levels of quinolinic acid and kynurenine-3-monooxygenase-activity tended to be higher in LC patients compared to the Ctrl, while tyrosine tended to be lower in the LC patients (Figure 4C, G and H). When comparing the LC cohort to the ME/CFS cohort, significant differences were observed: Specifically, quinolinic acid levels were higher in the LC group (median: 25.3) compared to the ME/CFS group (median: 17.6) (P-value = .02). Similarly, the kynurenine to tryptophan ratio was higher in the LC group (median: 37.7) compared to the ME/CFS group (median: 17.6) (P-value = .04). Additionally, neopterin concentrations were higher in the LC group (median: 1.6) compared to the ME/CFS group (median: 1.0) (P-value = .05) (Figure 4F, H and I).

However, it was observed that the individual patients with LC exhibited highly distinct metabolic profiles, suggesting diverse regulation of the depicted biochemical pathways in each patient. This variability in metabolic profiles is further detailed in Figure 5, which provides information on the number of study participants with elevated or decreased values of the specific metabolites.

Our findings revealed dysregulation of Vitamin B cofactors in the study cohorts. Specifically, we observed a significant reduction in nicotinic acid (Vitamin B3) levels in the ME/CFS cohort compared to the LC cohort. Moreover, the LC cohort showed lower methylmalonic acid concentrations compared to healthy Ctrl cohort (which means an adequate supply of vitamin B12) probably, because nearly all patients with LC also took supplements containing a B vitamin complex. Otherwise, no significant differences were seen between the different groups of the study population (Table 3).

Dopamine was increased in 14 out of 25 LC patients (56%), while only 2 of 8 patients in the ME/CFS cohort had increased dopamine levels (25%). Also 6 of 8 Ctrl had elevated levels of dopamine (75%). The exact numbers showing how many study participants had elevated values of the individual metabolites are shown in Figure 6.

Association between symptom scores and neurotransmitters

The correlation analyses revealed significant associations between the scores of various questionnaires. The PEM score showed a significant correlation with SSS-8 and SSD-12 scores. Furthermore, higher SSS-8 and SSD-12 scores were also associated with higher anxiety (GAD7 scores; $R = -.480$, P-value = .003 for SSS-8, $R = -.552$, P-value = $<.001$ for SSD-12). Additionally, higher SSS-8 and SSD-12 scores were correlated with more depressed mood (PHQ-9: $R = .468$, P-value = .003, .493, P-value = .001 for SSD-12).

A noteworthy finding was the significant positive correlation ($R = .463$, P-value = .003) observed between the PEM

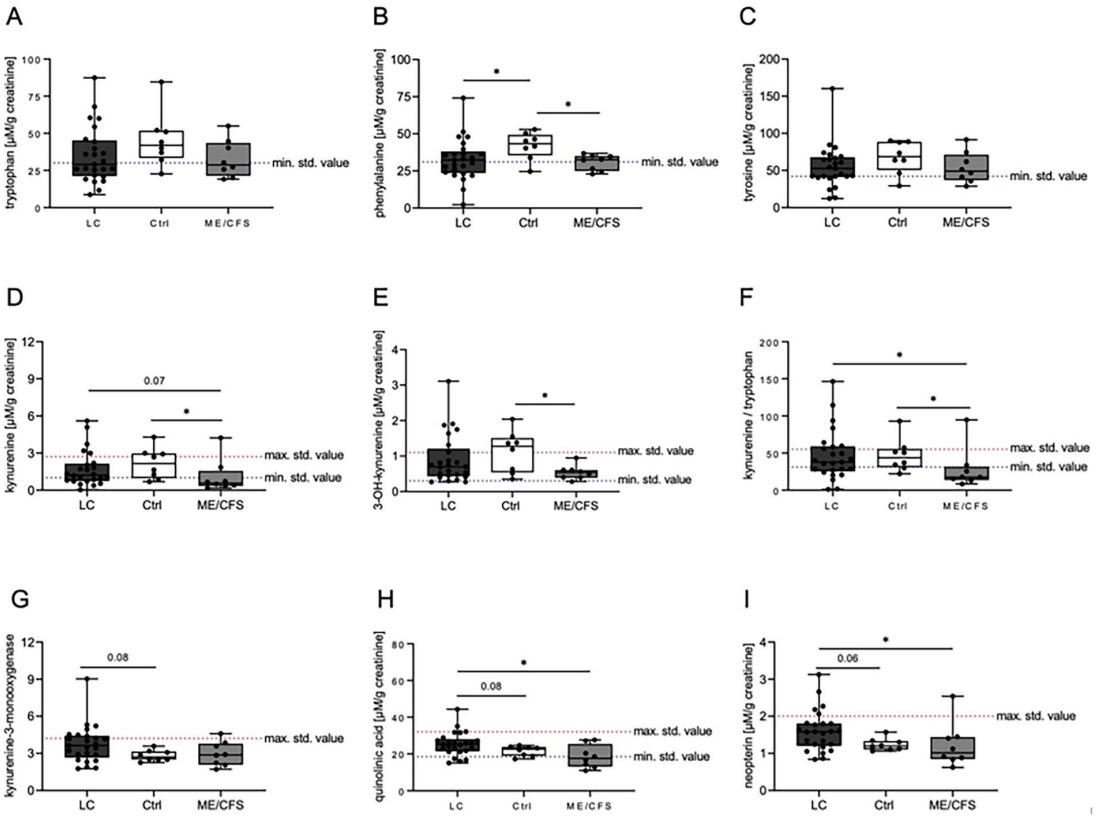

Figure 4. Concentrations of IFN-γ-induced metabolic pathways metabolites, enzymes and ratios analysed in urine of the study population. Concentrations of tryptophan (A), phenylalanine (B), tyrosine (C), kynurenine (D), 3-OH-kynurenine (E), ratio of kynurenine to tryptophan (F), kynurenine-3-monooxygenase-activity (G), quinolinic acid (H) and neopterin (I) measured in the urine of study participants suffering from Long-COVID (LC; black), healthy controls (Ctrl, white) and subjects diagnosed with ME/CFS (grey).
Significant differences were determined by Mann-Whitney *U* test. The maximum standard value of n > 100 participants (healthy according to self-report) based on the percentile distribution are depicted as a dashed red line. The minimum standard value of n > 100 participants (healthy according to self-report) based on the percentile distribution are depicted as a dashed blue line.
**P*-value <.05.

Table 2. Median of interferon gamma (IFN-γ) -induced metabolic pathways metabolites, enzymes and ratios in the study population.

	REFERENCE RANGE CORRECTED TO GRAM CREATININE	MEDIAN LC COHORT	MEDIAN CTRL COHORT	*P*-VALUE[a]	MEDIAN ME/CFS COHORT	*P*-VALUE[b]	*P*-VALUE[c]
Tryptophan	>30 µM	29	42	n.s.	28.7	n.s.	n.s.
Kynurenine	1-2.7 µM	1.2	2.2	n.s.	0.6	**.04**	.07
Kynurenic acid	>6.2 µM	6.9	8.6	n.s.	6.5	n.s.	n.s.
3-OH-Kynurenine	0.3-1.1 µM	0.7	1.3	n.s.	0.5	**.04**	n.s.
Quinolinic acid	18.5-32 µM	25.3	22.7	.08	17.6	n.s.	**.02**
Phenylalanine	>31 µM	32.3	43.4	**.03**	32.6	**.01**	n.s.
Tyrosine	>42 µM	52.8	68.6	.09	49.1	n.s.	n.s.
Kynurenine/Tryptophan	31-55	37.7	43.8	.08	17.57	**.03**	**.04**
Kynurenine-3-monooxygenase	<4.2	3.63	2.7	.08	2.87	n.s.	n.s.
Phenylalanine/Tyrosine	0.5-0.9	0.6	0.6	n.s.	0.6	n.s.	n.s.
Neopterin	<2 µM	1.6	1.2	.06	1.0	n.s.	**.05**

Significant *P*-values are printed in bold, not significant P-values are shown as n.s.
[a]Significant differences between the LC cohort and the healthy Ctrl cohort.
[b]Significant differences between the ME/CFS cohort and the healthy Ctrl cohort.
[c]Significant differences between the LC cohort and the ME/CFS cohort.

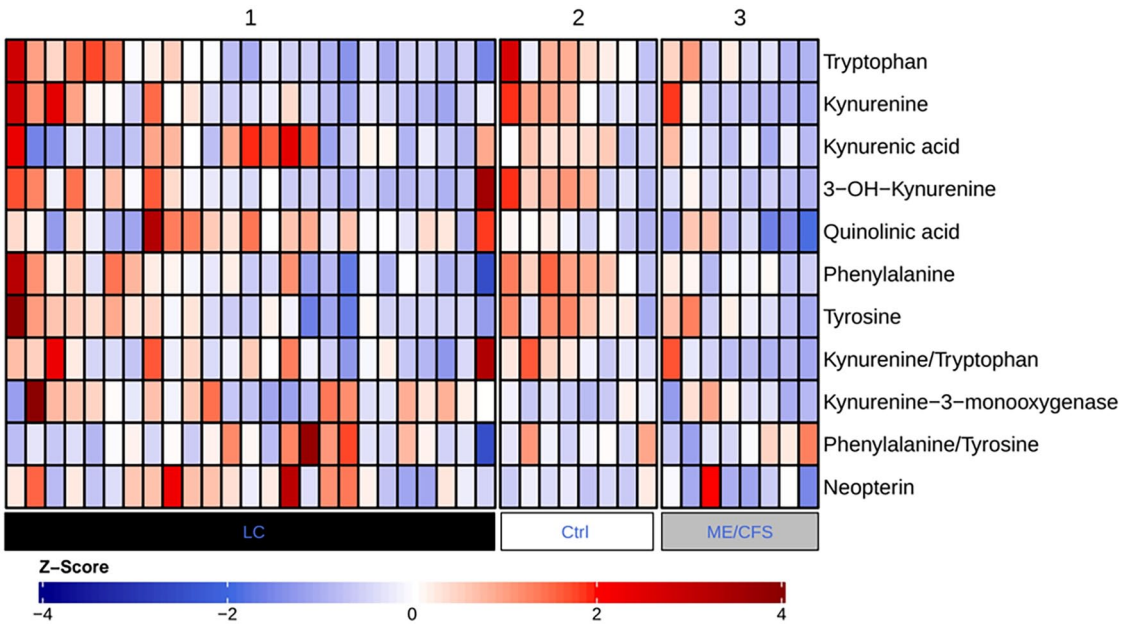

Figure 5. Heat map analysis of IFN-γ-induced metabolic pathways metabolites, enzymes and ratios in the study population. The row displays metabolite and the column represents the samples. Increased metabolites are displayed in red, and decreased metabolites in blue. The brightness of each colour corresponded to the magnitude of the difference when compared with average value.

Table 3. Medians of co-factors for neurotransmitter synthesis and neurotransmitter metabolites in the study population.

	REFERENCE RANGE CORRECTED TO GRAM CREATININE (μM)	MEDIAN LC COHORT	MEDIAN CTRL COHORT	*P*-VALUE[a]	MEDIAN ME/CFS COHORT	*P*-VALUE[b]	*P*-VALUE[c]
Dopamine	130-240	280	259.8	n.s.	215.9	n.s.	n.s.
Serotonin	80-190	95.8	94.1	n.s.	95	n.s.	n.s.
GABA	1.5-5.0	2.6	2.2	n.s.	3.1	n.s.	n.s.
Glutamate	8-25	8.4	7.7	n.s.	7.5	n.s.	n.s.
Nicotinic acid	>0.5	0.8	0.8	n.s.	0.5	.06	**.02**
NAD	>42	63.3	80.7	n.s.	52.1	n.s.	n.s.
Cystathionine	<25	7.8	5.2	n.s.	7.5	n.s.	n.s.
Methylmalonic acid	<1.8	1.2	1.8	**.02**	1.1	n.s.	n.s.

Significant *P*-values are bold, not significant *P*-values are shown as n.s.
[a]Significant differences between the LC cohort and the healthy Ctrl cohort.
[b]Significant differences between the ME/CFS cohort and the healthy Ctrl cohort.
[c]Significant differences between the LC cohort and the ME/CFS cohort.

score and glutamate levels (Figure 7A), suggesting an association between elevated glutamate levels and the severity of post-exertional malaise symptoms).

Correlation analysis also revealed that phenylalanine levels were correlated with somatic symptoms (SSS-8 scores ($R = -.343$, P-value = .035)) (Figure 7B) and tendentially with SSS-8 severity ($R = -.308$, P-value = .06).

Lower GABA concentrations were associated with higher SSS-8 scores ($R = -.322$, P-value = .049) and tended to coincide with a more depressed mood (PHQ-9: $R = -.304$, P-value = .056). Low dopamine levels and higher PHQ-9 levels also tended to be correlated ($R = -.291$, P-value = .076).

Correlation/associations of symptoms and metabolite profiles

We also analysed, whether the presence of various symptoms (Symptoms of the CCC, SSD-12, anxiety attacks, trauma

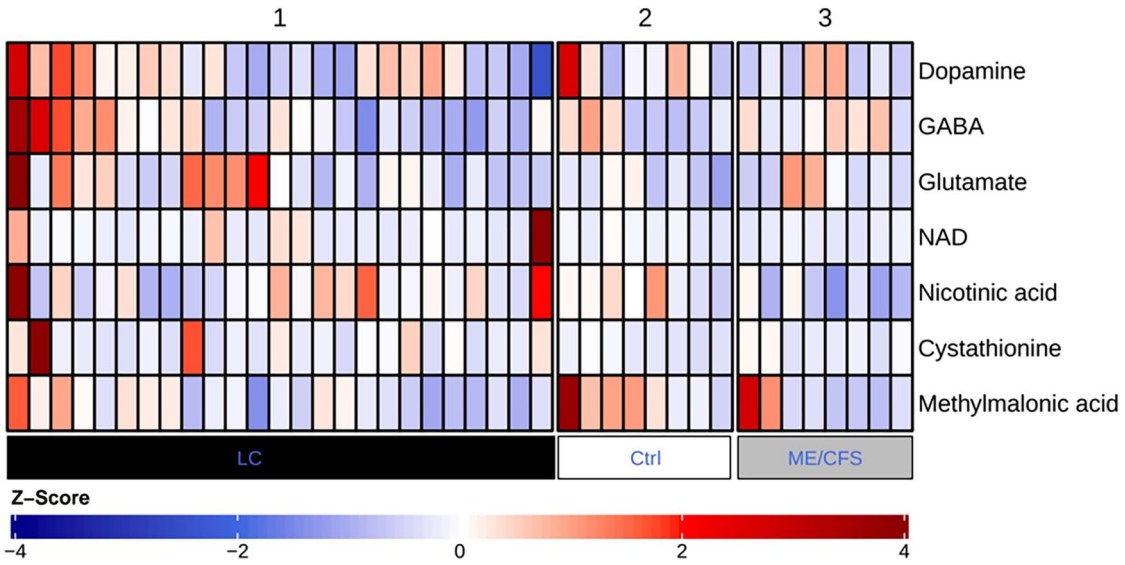

Figure 6. Heat map analysis of co-factors for neurotransmitter synthesis and neurotransmitters metabolites in the study population. The row displays metabolite and the column represents the samples. Increased metabolites are displayed in red, and decreased metabolites in blue. The brightness of each colour corresponded to the magnitude of the difference when compared with average value.

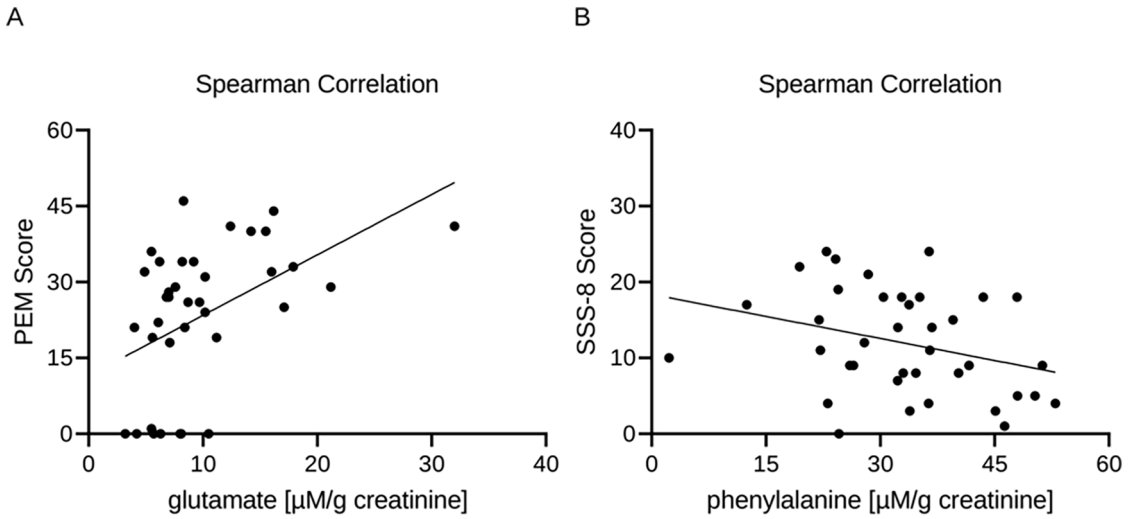

Figure 7. Correlation analysis of phenylalanine and symptom scores in the study cohort. (A) PEM score and glutamate concentration. (B) SSS-8 score and phenylalanine concentration.

experience) of the whole study population and especially the LC patients was related with alterations of neurotransmitter synthesis/metabolism of the amino acids tryptophan and phenylalanine.

Patients who reported to have fatigue (according to the CCC, n = 34) presented with lower noradrenaline levels than patients without fatigue (median 25.9 vs 35.3 µM, $P = .044$), furthermore also creatinine concentrations were significantly lower (median 118.6 vs 54.7 µM, $P = .008$) and phenylalanine levels tended to be lower (32.6 vs 41.6 µM, $P = .057$) in these patients. Interestingly, patients with PEM-scores >10 had lower kynurenine (median 1.06 vs 1.97 µM, $P = .049$) and lower phenylalanine levels (median 32.8 vs 40.9 µM, $P = .017$) as well as a decreased kynurenine/tryptophan ratio (median 36.0 vs 42.4, $P = .039$), kynurenic acid levels tended to be lower (median 6.47 vs 8.19 µM, $P = .052$).

The symptom sleep disorder of the CCC criteria affected 34 of 41 study participants. Patients with sleep disturbance had lower kynurenic acid concentrations compared to patients with good sleep (median of 6.5 vs 8.7 µM, P-value = .044). Similarly, nicotinic acid (0.75 vs 1.0 µM, $P = .043$) and neopterin were lower in patients with sleep disorder (median 1.3 vs 1.8 µM, $P = .061$). Methylation index (SAM/SAH Ratio) was significantly higher in subjects affected by sleep disorder (median 10.2 vs 8.5, P-value = .041).

In the LC group, 21 out of 25 participants reported poor sleep quality, which was associated with lower concentrations of quinolinic acid and nicotinic acid compared to those who

Table 4. Medians of IFN-γ-induced metabolic pathways metabolites and co-factors for neurotransmitter synthesis and neurotransmitters metabolites in LC patients with/without sleep disturbances.

	REFERENCE RANGE CORRECTED TO CREATININE	LC PATIENTS WITH GOOD SLEEP	LC PATIENTS WITH SLEEP DISTURBANCE	*P*-VALUE[a]
Kynurenine	1-2.7 µM	2.1	1.0	.054
Kynurenic acid	>6.2 µM	10.2	5.6	.075
Quinolinic acid	18.5-32 µM	31.7	24.1	**.012**
Neopterin	<2 µM	2.2	1.5	**.008**
Nicotinic acid	>0.5 µM	1.2	0.8	**.013**
SAM to SAH ratio	2.2-6.4 nmol/L	5.5	10.3	**.018**

Significant *P*-values are bold and *P*-values with a tendency towards being significant are depicted.
[a]Differences between LC patients with (n = 21) and without sleep disturbance (n = 4).

Table 5. Medians of IFN-γ-induced metabolic pathways metabolites and co-factors for neurotransmitter synthesis and neurotransmitters metabolites in individuals with/without symptoms of autonomic dysfunction.

	REFERENCE RANGE	PATIENTS WITHOUT AUTONOMIC DYSFUNCTION	PATIENTS WITH AUTONOMIC DYSFUNCTION	*P*-VALUE[a]
Kynurenine	1-2.7 µM	2.7	1.0	**.025**
Kynurenic acid	>6.2 µM	8.8	6.1	**.023**
3-OH-Kynurenine	0.3-1.1 µM	1.4	0.6	.083
Kynurenine/Tryptophan	31-55	51.6	31.1	**.041**
Kynurenine-3-monooxygenase	<4.2	2.5	3.6	.095
Phenylalanine	>31 µM	45.1	32.3	**.002**
Tyrosine	>42 µM	68.7	49.1	.078
Quinolinic acid	18.5-32 µM	6.2	10.1	.095
Nicotinic acid	>0.5 µM	0.9	0.7	.061
Methylmalonic acid	<1.8 µM	2	1.2	**.013**

[a]Significant differences between patients with and without symptoms of autonomic nervous system dysfunction. Significant *P*-values are kept in bold.

experienced refreshing sleep. Additionally, there was a tendency towards lower levels of kynurenine and kynurenic acid in the poor sleep group (see Table 4).

Out of the 41 participants in the study population, 34 reported experiencing pain. Creatinine levels were lower in patients with pain ($P = .04$), and also noradrenaline tended to be lower (median 35.3 vs 25.9 µM, $P = .083$). In the LC cohort, the following trends were seen: cystathionine levels tended to be lower in patients with pain (n = 23, median 6.9 vs 34.8 µM in pain-free patients, $P = .089$), while tryptophan and 3-OH-kynurenine tended to be higher (Tryptophan: median 31.2 vs 18.3 µM; *P*-value = .071; 3-OH-kynurenine: median 0.7 vs 0.35 µM, *P*-value = .089).

Patients who reported neurological symptoms (n = 35, 2 Ctrl also stated that they sometimes had problems with their concentration/memory) had lower creatinine (55.4 vs 132.2 µM, $P = .013$) and higher methylmalonic acid levels (median 1.84 vs 1.17 µM, $P = .046$), also noradrenaline levels tended to be lower (median 25.9 vs 35.2 µM, $P = .08$).

Among the 41 study participants, 32 reported symptoms of autonomic nervous system dysfunction (at least one symptom of the CCC criteria: orthostatic hypotonia/drowsiness, dizziness, postural tachycardia, extreme paleness, intestinal disorders [diffuse abdominal pain, bloating, burning sensations], bladder disorders, palpitations, dyspnoea with light exertion). Interestingly, we observed differences in the analysed metabolites, as shown in Table 5.

Similarly, 23 out of 25 patients suffering from LC were affected. Levels of kynurenine, kynurenic acid, quinolinic acid, Kynurenine/Tryptophan ratio, nicotinic acid and neopterin tended to be slightly lower in affected subjects (all *P*-values <.1).

In the study population, symptoms of neuroendocrine dysfunction (n = 36) according to the CCC were associated with lower phenylalanine levels compared to participants without symptoms (median 32.9 vs 45.1 µM, *P* = .067).

Furthermore, symptoms of immunological impairment (n = 28) according to the CCC were linked to slightly lower dopamine concentrations compared to unaffected participants (median 228.1 vs 293.6 µM, *P* = .06). Notably, in LC patients, dopamine levels were significantly lower in those with symptoms (median 225.1 vs 312.1 µM, *P* = .049).

High psychosocial stress levels (SSD-12 score >20 points, n = 18) were associated with a lower phenylalanine/tyrosine ratio in the LC cohort compared to participants with lower psychosocial stress (median 0.5 vs 0.7 µM/mM, *P* = .022). Additionally, kynurenic acid was significantly lower in the group with an SSD-12 score over 20 (median = 5.5) compared to the group with lower stress scores (median = 8.6, *P*-value = .03).

Anxiety attacks (n = 14) were correlated with higher kynurenine-3-monooxygenase (KMO) ratios compared to participants without this symptom (median 4.22 vs 2.87 µM, *P* = .002). GABA levels were lower in the 14 participants with anxiety compared to unaffected participants (median 1.97 vs 2.93 µM, *P* = .039). Similarly, LC patients with anxiety attacks (n = 13) had higher KMO ratios (median 4.3 vs 3, *P* = .007), and kynurenic acid levels were significantly lower in LC patients with anxiety attacks (median 5.5 vs 10.2 µM, *P* = .04).

Patients who had been exposed to traumatic events (according to the DSM, n = 17) displayed significantly lower dopamine, noradrenaline and adrenaline levels compared to non-traumatized individuals (Dopamine median 195.1 vs 184.8 µM, *P* = .003; adrenaline: 3.1 vs 6.2 µM, *P* = .006; noradrenaline: 23.4 vs 34.0 µM, *P* = .02).

Patients with depression (GAD-7 score >5, n = 12) tended to have lower kynurenic acid (median 5.5 vs 7.6, *P*-value = .068) and a lower GABA/Glutamate ratio (median 0.25 vs 0.35; *P*-value = .094).

Discussion

Patients suffering from LC are very often affected by multiple symptoms like fatigue, pain, neurological symptoms and symptoms of autonomic and neuroendocrine dysfunction, immune disorders as well as anxiety and depression. This was also true for the patient cohort of this proof of principle study. Similar to patients suffering from ME/CFS their quality of life is frequently reduced, and post-exertional malaise significantly impairs their ability to work or even take care for themselves. In our cohort, nearly all patients with LC and ME/CFS suffered from severe fatigue, and presented with higher levels of stress and somatic symptoms compared to healthy individuals. Patients with LC also reported more anxiety attacks and had higher depression scores.

In this study, we describe results of urine metabolite analyses in patients with LC and ME/CFS compared to healthy controls. The non-invasive determination of levels of amino acids and patients' urine metabolites, may be useful to depict important biochemical pathways activities like tryptophan catabolism and catecholamine formation, and thus may be helpful to investigate underlying pathomechanisms.

Similar to the study by Rosolanka et al[46] we analysed concentrations of urine metabolites related to urine creatinine levels of Long-Covid patients and healthy controls. Compared to this study we used acid stabilized second morning urine and focused on IFN-γ-induced metabolic pathways metabolites, co-factors for neurotransmitter synthesis and neurotransmitters metabolites at a single examination date. Furthermore, we compared the levels of metabolites to healthy Ctrls and patients suffering from ME/CFS. Rosolankea et al were able to show that reconvalescent Covid patients have elevated tyrosine levels and postulate that these levels can arise from either excessive production from phenylalanine or inadequate utilization.[46] They propose that individuals afflicted with acute severe COVID-19 experience reduced tyrosine utilization, potentially leading to decreased synthesis of thyroid hormones and neurotransmitters like dopamine and noradrenaline.[46] In this study we could show that phenylalanine levels were decreased in LC patients compared to healthy Ctrls going along with slightly decreased tyrosine levels. Concerning neurotransmitter synthesis, dopamine levels were tendentially higher in LC patients, suggesting that tyrosine utilization was not reduced in patients with LC symptoms, rather the catabolism of catecholamines appeared to be impaired in many patients- maybe caused by polymorphisms of the enzyme Catechol-O-methyltransferase (COMT).

Earlier studies have shown that patients with ME/CFS have elevated immune activity, oxidative stress and impaired neurotransmitter production.[47] As patients with cancer or infections were demonstrated to have immune-mediated changes of tryptophan and phenylalanine metabolism and are therefore more likely to feel fatigued and depressed,[32,48-51] we postulate that changes in IFN-γ mediated pathway metabolites and changes in neurotransmitter synthesis might also be crucially involved in LC and ME/CFS patients and that such changes are traceable in urine.

As proof of the concept, we found significant alterations of these biochemical pathway profiles in the urine of patients, phenylalanine levels were significantly lower in patients with LC and ME/CFS than in healthy individuals. In addition, tryptophan and tyrosine levels were lower (albeit not significantly), and decreased levels of downstream metabolites of tryptophan (ie, kynurenine, 3-OH kynurenine and kynurenic acid) which were seen in nearly half of patients indicate impaired metabolism along the kynurenine pathway. These dysbalances may result in decreased formation of nicotinic acid and NAD, one of the most important 'fuels' for mitochondria.

Patients suffering from post-infectious fatigue (ie, LC and ME/CFS) presented with significantly lower noradrenaline concentrations, lower creatinine levels and a tendency towards lower phenylalanine levels. In patients with higher PEM scores, lower concentrations of downstream metabolites like kynurenine, and kynurenic acid were found, furthermore, phenylalanine concentrations and kynurenine/tryptophan ratio were lower. These data suggest, that fatigue severity is related to adrenal function and metabolic alterations of tryptophan and phenylalanine metabolism. Furthermore, higher PEM scores were also related with higher glutamate levels, indicating a shift of neurotransmitter balance to over-excitation. Also, lower levels of neuroprotective substances like kynurenic acid and GABA in patients with sleep disturbances and anxiety, respectively, indicate dysbalances of important neurotransmitters in patients with these symptoms. Higher quinolinic acid concentrations and KMO activity in LC patients also fit well with this hypothesis.

B-vitamins are often used by patients with fatigue to improve neurotransmitter balance, memory and energy levels. This was also the case in our study: As most LC and also ME/CFS patients took vitamin B supplements and coenzyme Q10, we suppose that the observed impairments in neurotransmitter and NAD formation might have been more pronounced otherwise. We only observed decreased NAD levels in a small subset of 7 patients, and LC patients even had lower methylmalonic acid levels compared to healthy individuals, indicating adequate vitamin B12 levels in LC patients and low B12 levels in healthy individuals.

Compared to ME/CFS patients, LC patients showed higher levels of kynurenine/tryptophan ratio and quinolinic acid. There is evidence that the N-methyl D-aspartate (NMDA) receptor agonist and neurotoxin quinolinic acid plays a role in many psychiatric disorders, as well as neurodegenerative disorders in the brain.[52-54] LC patients presented with significantly higher quinolinic acid concentrations than patients with ME/CFS and tended to have higher levels as healthy controls, which fits well with our observations, that LC patients often appear to suffer from 'inner restlessness'-which might be due to higher levels of excitatory neurotransmitters in relation to neuroprotective neurotransmitters (like GABA or kynurenic acid). Well in line with this hypothesis, GABA concentrations were lower in patients with anxiety, while KMO-activity was significantly higher in anxious patients and kynurenic acid tended to be lower in LC patients with anxiety.

Patients with depression also had slightly lower kynurenic acid and a lower GABA to glutamate ratio, earlier enhanced KMO-activity has been shown to go along with inflammation-mediated dysregulation of the kynurenine pathway.[55] In patients with acute central nervous system infections parallel increases of metabolites of the kynurenine pathway (3-OH-kynurenine, quinolinic acid, picolinic acid, kynurenic acid) in the CSF and serum went along with enhanced neopterin production and higher neopterin was indicative of a shift to more neurotoxicity.[56]

Interestingly, the LC cohort also showed higher neopterin levels compared to ME/CFS suggesting increased Th1 type immune activation and disease burden in the LC cohort. Disease activity and extent have been correlated with elevated levels of neopterin also in other diseases,[57-59] neopterin appears to be higher mainly in patients suffering from autoimmune and cancer disease.[31,32] A recent meta-analysis also reported that neopterin concentrations were higher in patients with major depression, both in patients with and without medication.[60]

Earlier, higher kynurenine/tryptophan ratios were associated with augmented depressive and anxiety symptoms,[61] in our patients with LC, however, kynurenine concentrations were lower. In line with that result, a trial with participants suffering from CFS found a decreased kynurenine to tryptophan ratio and lower kynurenine levels compared to healthy controls, which could be due to a lower availability of the substrate tryptophan-probably by impaired gut function-or an impaired function of IDO.[62,63]

Decreased absorption of essential amino acids like tryptophan, phenylalanine and tyrosine in the gut or the enhanced immune mediated catabolism along the kynurenine pathway consecutive to gut dysbiosis and inflammation might also be limiting for the formation of important neurotransmitters. Consequently, this is known to cause low blood tryptophan, phenylalanine and tyrosine concentrations, which are the precursors of serotonin, dopamine and catecholamines. Decreased levels of downstream metabolites like kynurenine, OH-kynurenine, kynurenic acid and nicotinamide in combination with low levels of tryptophan, tyrosine and phenylalanine most probably reflect reduced absorption of these essential amino acids by the gut, which might be due to enhanced consumption of amino acids by proteobacteria.

Reduced blood phenylalanine and tyrosine levels could also be due to catabolic conditions (eg, by cachexia) or to accumulation of downstream metabolites: As many patients showed high dopamine and some also high catecholamine concentrations, feedback-regulated processes might also explain low levels of amino acids phenylalanine and tyrosine.

Patients with low catechol-o-methytransferase (COMT)-activity have an impaired ability to degrade catecholamines, thus often presenting with high levels of dopamine and catecholamines-which goes along with enhanced focus and concentration, but also enhanced sensitivity to stimuli (which is often a problem in patients with LC or ME/CFS). Microbial beta glucuronidases, which can be decreased or elevated in patients with dysbiosis, might modulate COMT activity by interfering with hormone metabolism: Oestrogen lowers COMT activity[64] while testosterone enhances COMT activity.[65] Lower SAH/SAM ratios in patients with

sleep disturbance and more than 10 symptoms might also be a hint, that methylation could be impaired in a subset of patients.

Our study has several limitations that should be acknowledged. Firstly, it was conducted as a single-centre study, resulting in a limited sample size for our cohort. Although we initially collected urine metabolome samples from a larger number of patients (32 patients with LC, 12 Ctrl, and 12 patients with ME/CFS), we could only include results from patients whose urine creatinine levels fell within the reference range. Secondly, due to recruitment constraints, we only had urine metabolome profiles of 8 healthy controls, making it impossible to achieve sex-matching. Similarly, we could only obtain urine metabolite data of 8 ME/CFS patients, and their gender was not matched to the LC cohort or the Ctrl. Conducting further studies with larger participant numbers and improved sex matching would be valuable.

In summary, our pilot study analysed urine samples from LC patients, ME/CFS patients and Ctrl to investigate IFNγ mediated pathway metabolites and neurotransmitter synthesis. This approach revealed signatures of altered metabolism in a hitherto 'untreatable' disease, thus providing a basis for further research. The observed dysregulation of biochemical pathways in LC patients may vary depending on factors such as the extent of dysbiosis, specific bacterial subspecies involved, genetic predisposition (eg, polymorphisms of catechol-o-methyltransferase or monoamine oxidase, or polymorphisms of immune system genes) and environmental influences. These factors could explain the heterogeneity of symptoms and their varying severity among LC patients. Preliminary data from our urine metabolome analyses suggest that diagnostic metabolomic approaches have the potential to identify diverse underlying pathogenetic mechanisms using a single sample. The observation that patients with different symptoms also exhibit varying concentrations of the investigated metabolites, likely influenced by gut function, gut microbial composition, genetic and psychosocial factors, and possibly other environmental and personal stressors, highlights the need for larger cohort studies. Such studies can help identify patient subgroups and enable personalized and targeted therapies based on altered metabolic signatures.

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Author Contributions

Investigation: A.S., M.T., N.B., K.K.; resources: J.L-R., K.K.; data curation: M.T., N.B.; Software: N.B., M.T., P.M.-L.; Formal analysis: S.E., N.B., P.M.-L., K.K.; Writing - original draft preparation: N.B., M.T., K.K.; writing - review and editing, J.L-R., A.S., M.T., S.E. and G.W.; visualization: N.B.; supervision: G.W., K.K.; project administration: N.B., K.K.; funding acquisition: J.L.-R., K.K., G.W. All authors have read and agreed to the published version of the manuscript.

Institutional Review Board Statement

The study was conducted in accordance with the Declaration of Helsinki and approved by the ethical committee of Innsbruck Medical University (ID of the Ethical vote: EK-Nr 2017/1157; EK-Nr 1103/2020).

Informed Consent Statement

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

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Data Availability Statement

Results presented within this study are available within the manuscript. Blinded raw data are available upon request.

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