Gastrointestinal barrier disruption in Post-COVID Syndrome Fatigue patients

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Abstract

Background: Post-COVID-Syndrome (PCS) is the term for a condition with persistent symptoms in a proportion of COVID-19 patient after asymptomatic, mild or severe disease courses. Numbers vary but the current estimate is that after COVID-19 approximately 10% develop PCS. The aim of our study was to evaluate the impact of SARS-CoV-2 infection on the gastrointestinal (GI) tract and associations with the development of PCS with fatigue, post-exertional malaise (PEM), orthostatic dysregulation, autonomous dysregulation and/or neurocognitive dysregulation. **Methods:** By combining medical record data from a prospective observational study with symptom analysis before, during, and after SARS-CoV-2 infection, we aimed to identify potential risk factors and predictive markers for PCS. Additionally, we analyzed blood, saliva, and stool samples from this well-characterized PCS patient cohort to biologically validate our findings. **Results:** We identified significant associations between pre-existing GI complaints and the development of PCS Fatigue. PCS patients showed higher LBP/sCD14 ratios, lower IL-33 levels, and higher IL-6 levels compared to control groups. Our results highlight the critical role of the GI tract in PCS development of post-viral Fatigue. **Conclusion:** We propose that the viral infection disrupts pathways related to the innate immune response and GI barrier function, evidenced by intestinal low-grade inflammation and GI barrier leakage. Monitoring GI symptoms and markers before, during and after SARS-CoV-2 infection is crucial for identifying predictive clinical phenotypes in PCS. Understanding the interaction between viral infections, immune responses, and gut integrity could lead to more effective diagnostic and treatment strategies, ultimately reducing the burden on PCS patients.

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Main text file

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Short Title: Gut Barrier Disruption in Post-COVID Fatigue

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Methods: By combining medical record data from a prospective observational study with symptom analysis before, during, and after SARS-CoV-2 infection, we aimed to identify potential risk factors and predictive markers for PCS. Additionally, we analyzed blood, saliva, and stool samples from this well-characterized PCS patient cohort to biologically validate our findings.

Results: We identified significant associations between pre-existing GI complaints and the development of PCS Fatigue. PCS patients showed higher LBP/sCD14 ratios, lower IL-33 levels, and higher IL-6 levels compared to control groups. Our results highlight the critical role of the GI tract in PCS development of post-viral Fatigue.

Conclusion: We propose that the viral infection disrupts pathways related to the innate immune response and GI barrier function, evidenced by intestinal low-grade inflammation and GI barrier leakage. Monitoring GI symptoms and markers before, during and after SARS-CoV-2 infection is crucial for identifying predictive clinical phenotypes in PCS. Understanding the interaction between viral infections, immune responses, and gut integrity could lead to more effective diagnostic and treatment strategies, ultimately reducing the burden on PCS patients.

Keywords (5/5): post-COVID syndrome, post-viral fatigue, gastrointestinal barrier function, immune barrier, predictive phenotypes

Main Text

1. Background

Due to the Post-COVID Syndrome (PCS), health care systems worldwide are confronted with an increasing number of patients who do not fully recover after SARS-CoV-2 infection [1, 2]. The exact pathomechanism is not yet understood and appears to vary individually. A portion of patients affected by PCS reports a clinical picture that shows significant overlap with the chronic, multisystem disease Myalgic Encephalomyelitis/Chronic Fatigue Syndrome (ME/CFS) [1, 2]. Since there is no clinically validated biomarker for either condition and the diagnosis often relies on clinical symptoms [3], the exact number of affected individuals can only be estimated. In the case of ME/CFS, a prevalence of 0.3 to 0.8% is assumed [4]. We know that a subgroup (1-10%) of individuals previously infected with SARS-CoV-2 might develop PCS of the ME/CFS type, which is expected to double the prevalence of ME/CFS in the coming years [1, 2]. Similar to PCS, ME/CFS is often reported to begin with viral infections [5, 6]. Both conditions are suspected to involve altered immune functions such as chronic immune activation or immunodeficiencies, but also gastrointestinal dysregulation, mitochondrial dysfunctions and metabolic changes are described [5, 7-10]. In many cases, a recruitment of immune cells and the subsequent enhanced release of cytokines is suspected to detrimentally effect the otherwise tightly regulated pathways in the body.

Although, SARS-CoV-2 primarily targets the lungs causing respiratory symptoms, the infection affects multiple organ systems, including the GI tract. SARS-CoV-2 can cause direct injury to intestinal epithelial and endothelial cells or induce damage indirectly through immune responses. Significant changes to the intestinal microbiota were observed, disrupting local immune responses [7]. Additionally, it compromises the barrier's structural integrity by altering the expression of tight junction proteins [11]. Persistent SARS-CoV-2 reservoirs in host organs are plausible, as other respiratory RNA viruses, such as latent respiratory syncytial virus or influenza A virus, have been observed to persist for extended periods in murine models [12, 13]. The angiotensin converting enzyme 2 (ACE-2) expressed in the epithelium of the GI tract serves as an entry site and as a reservoir for SARS-CoV-2. A systematic review and meta-analysis of studies reported GI symptoms in patients after COVID-19 were seen in 12% of non-PCS patients and in 22% of PCS patients, suggesting an even more important role of the GI tract in PCS than during the acute infection [14]. This raises the question, whether viral persistence, organ damage during acute COVID-19 or other persistent GI changes might be observed with PCS.

Our study aims to address this issue by combining medical record data of a prospective observational study with analysis of blood, saliva and stool samples of a well characterized PCS patient cohort. We focus not only on symptoms experienced during and after the disease, but also on complaints before SARS-CoV-2 infection to identify potential risk factors and predictive markers.

2. Methods

2.1. Study Cohort

All participants were recruited between April 2021 and August 2022 and provided written informed consent before study inclusion (ethic vote number: 2281/2020). The study cohort consists of PCS patients, as well as sex- and age-matched SARS-CoV-2 convalescent participants (SARS-CoV-2, convalescent), SARS-CoV-2 naïve ME/CFS patients (ME/CFS) and SARS-CoV-2 naïve healthy participants (Healthy) as controls**(Table 1**). ME/CFS was diagnosed based on the Institute of Medicine (IOM) criteria [15]. All included ME/CFS patients suffered from an Epstein-Barr Virus (EBV)-related onset of the disease. For study participation, PCS patients had to suffer from Fatigue, PEM and additionally from clinical signs of autonomous dysregulation, orthostatic dysregulation and/or neurocognitive dysregulation. SARS-CoV-2 infected study participants had an asymptomatic or mild disease course, with 4 exceptions in the PCS group and 2 exceptions in the convalescent SARS-CoV-2 control group. Furthermore, previously SARS-CoV-2 positive participants were excluded if their acute COVID-19 diseases required intensive medical care. All participants were at least 10 weeks past an acute SARS-CoV-2 infection, at least 12 weeks past an acute EBV infection and at least 2 weeks after any other respiratory or GI infection. Participants were excluded if they suffered from preexisting malignant diseases, diabetes mellitus, inflammatory bowel diseases, an acute sepsis or if they underwent medical treatment with antibiotics, analgesics or antacids one month prior to study participation. PCR or SARS-CoV-2 antigen test was performed to screen for clinically inapparent SARS-CoV-2 infections prior to study inclusion.

2.2 Sampling Procedure and Self-reported Medical Record Data

Before the sampling, participants were asked to give a detailed medical record on their disease course and individual symptoms before, during and after the SARS-CoV-2 infection, or their general health status for the SARS-CoV-2 naïve control participants (**Table 2** ,**Supplementary Table 1 & 2**). Serum and plasma/EDTA samples were collected on the day of study participation, after centrifugation with 2000× g for 10 min. Stool samples were collected and delivered by the patients on the day of study participation. Participants were asked to cool their samples in the fridge for storage prior to delivery. If the collection of a stool sample was not possible on the day of study participation, it was collected by the patient within two weeks after study participation. Stool extracts for further use were prepared according to the respective manufacturer's protocols (**Supplementary Table 3**). Saliva and throat flushing samples were collected by the patients on the day of study participation, before tooth brushing and eating or drinking. Saliva samples were collected pure, throat flushing samples were collected by gurgling for 1 minute with 10 ml 0.9% NaCl.

All samples were stored at -20°C until further processing.

2.3 SARS-CoV-2 detection in blood, stool and saliva samples

SARS-CoV-2 viral load in body fluids was evaluated by real-time RT-PCR using primers targeting the SARS-CoV-2 E-gene as previously published [16, 17]. Briefly, RNA was extracted from stool, plasma and saliva samples using the NucliSens EasyMag extractor system (BioMérieux, Marcy-l'Étoile, France). SARS-CoV-2 RNA was eluted and a reaction was prepared with the Superscript III one step RT-PCR system with Platinum Taq Polymerase (Invitrogen, Darmstadt, Germany) and reverse transcriptase/Taq mixture from the kit. Thermal cycling was performed at 55 degC for 10 min for reverse transcription, followed by 95 degC for 3 min and then 45 cycles of 95 degC for 15 s, 58 degC for 30 s.

2.4 Measurement of disease-related parameters.

Parameters for evaluating SARS-CoV-2 specific immune responses, systemic and local inflammation and intestinal barrier disruption were measured by commercially available Enzyme-linked immunosorbent assays (ELISAs). All markers and respective ELISA Kits are listed in the supplementary materials (**Supplementary Table 3**). ELISAs were performed according to the respective manufacturer's protocols. Absorbance was measured at 450 nm using ELISA Reader Infinite m200 PRO (Tecan, Mannedorf, Switzerland). A four-parameter logistic (4PL) curve was used to analyse antibody concentrations after subtracting levels detected in blank wells as background values.

2.5 Statistical analysis

Data sets were tested for normal distribution with Kolmogorov-Smirnov test. Significance between groups was assessed by Kruskal-Wallis-test and Dunn-Bonferroni-test for multiple comparison were performed. Anamnestic data was described using descriptive statistical methods. Characteristics were either given in absolute numbers or percentages for categorical variables or in medians/quartiles for continuous variables. To investigate possible associations between different biomarkers, cytokines and anamnestic characteristics and the development of PCS Fatigue chi-squared test (nominal and nominal variables) and Mann-Whitney-U test (nominal and metric variables) were used. Univariate binary logistic regression was performed for all patients after a SARS-CoV-2 infection (PCS and SARS-Cov-2, convalescent) with "developed PCS – yes/no" being the binary, dependent variable and LBP, sCD14, I-FABP, IL-33 and zonulin family peptides being the independent variable. In advance, the data used for regression was screened for statistical outliers using the ROUT method, as well as linear correlation. Through binary logistic regression odds ratios (OR) and p-values were calculated. Two tailed p-value of <0,05 was taken as statistically significant. Statistical analyses were performed by using Graph Pad Prism 9 or IBM SPSS Statistics 27. No adjustment for multiplicity was performed, p-values were interpreted descriptively.

3. Results

3.1 GI complaints before, during and after acute SARS-CoV-2 infection is associated with PCS Fatigue

PCS and ME/CFS patients reported a higher susceptibility to infections (**Table 2**). A higher number of PCS and ME/CFS patients reported signs of hypermobility. Pre-existing food intolerances were more common in PCS and ME/CFS patients. Notably, only PCS patients had more pre-existing GI complaints before acute infection. A significant association between GI symptoms prior to SARS-CoV-2 infection compared to current GI complaints was detected (chi-square test value = 13.8; p<0.001). Also, a significant association was found between pre-existing GI symptoms and PCS development (chi-square test value = 5.9; p=0.015). During acute infection, PCS patients reported more often respiratory, cardiovascular and GI symptoms, but also head ache, myalgia, back pain, hair loss and sleep disorders (**Supplementary Table 1**). In general, multisystemic symptoms during acute infection were more commonly found in PCS patients compared to those who fully recovered. 100% of PCS patients still suffer from exercise intolerance, and 50% were unable to work. Significant association were also observed for GI complaints (chi-square test value = 6.6; p=0.01) and cardiovascular symptoms (chi-square test value = 9.8; p=0.002) during acute infection and their persistence in PCS. In terms of current symptoms, respiratory syndromes, neurological symptoms, cardiovascular

symptoms, GI complaints, pain symptoms, signs of cognitive impairment, dysautonomia symptoms, sleeping disorders, as well as signs of Fatigue and PEM (reduced mental and physical activity, ability to leave the house or go to work) occurred more often in PCS and ME/CFS patients. (**Supplementary Table 2**).

3.2 No signs of viral shedding were observed by determining SARS-CoV-2 RNA levels in body fluids

We evaluated SARS-CoV-2 E-protein RNA levels to examine viral shedding evidenced by viral RNA in body fluids. Plasma, stool and throat washing samples were analyzed by RT-qPCR, but no viral particles were found (data not shown).

3.3 Comparison of SARS-CoV-2 IgG and IgA antibody titers and immunological events in the study cohort

Measurements of SARS-CoV-2 specific IgG and IgA antibodies against S1/RBD in plasma by ELISA showed no statistical differences in IgG titers between the test groups. SARS-CoV-2 convalescent study participants had significantly higher IgA titers than the healthy control group (**Supplementary Figure 1A & 1B**). To include data on the participants' vaccination state and provide a better context of the results, anamnestic information on immunological events was analyzed (**Supplementary Figure 1C & 1D**). The term "immunological event" refers to either a SARS-CoV-2 infection or a SARS-CoV-2 vaccination with a single event in a 14-day period. No statistical differences were observed when comparing the time between the last immunological event and the day of study inclusion and sampling. SARS-CoV-2 convalescent participants had a higher number of immunological events, although, not significant.

3.4 PCS patients reveal signs of intestinal barrier leakage

PCS patients showed significantly higher serum lipopolysaccharide-binding protein (LBP) levels compared to convalescent SARS-CoV-2 participants and healthy controls (**Figure 1A**). Levels of sCD14 in serum samples were significantly lower compared to all other groups (**Figure 1B**). As sCD14 is needed as a co-factor together with LBP to mediate innate immunity against LPS to in the immune system, the LBP/sCD14 ratio was calculated. We were able to detect a significantly higher ratio in PCS patients compared to convalescent SARS-CoV-2 participants and healthy controls (**Figure 1C**). Serum I-FABP, which is released by enterocytes upon cell damage, did not differ between all groups (**Figure 1D**).

3.5 Evaluation of disease-related marker associated with low-grade inflammation and intestinal barrier disruption

To detect signs of chronic low-grade inflammation in PCS patients, the pro-inflammatory and intestinal barrier integrity- related cytokines IFN-γ, TNF-α, IL-6, IL-1-β, IL-8, and IL-33 were measured in serum samples. A significant elevation in IL-6 levels and a reduction in serum TNF-α and IL-1-β levels was observed in PCS patients (**Figure 2 A-C**). However, some results of the pro-inflammatory cytokine ELISAs were below the Limit of Detection (LOD) stated by the manufacturer and had to be excluded for the analysis. The means ($\pm$ SD) of the respective parameters' remaining values were under or close to the LOD (**Supplementary Table 4**). Serum IL-33 levels were significantly decreased in PCS patients when compared to the SARS-CoV-2 negative control participants (**Figure 2D**). IL-8 and IFN-γ levels did neither show statistical significance, nor a disease- specific pattern (data not shown).

3.6 No significant differences in fecal pro-inflammatory marker levels were detected

To assess whether participants show signs of intestinal inflammation levels of calprotectin, β-defensin-2, zonulin family peptides and serotonin (5-HT) were evaluated (**Table 3**) by ELISA. No significant differences between the groups were found when examining levels of calprotectin, β-defensin-2 and serotonin (5-HT).

3.7 Association analysis between the development of PCS and intestinal barrier marker

To assess influence of gastrointestinal barrier markers on the development of PCS, we used univariate binary logistic regression. Statistical outliers were removed from the analysis before calculating odds ratios (OR) and p-values (**Supplementary Table 5**). We analyzed all SARS-CoV-2 positive participants to investigate differences in intestinal barrier markers related to the development of PCS (binary, dependent variable). A

higher LBP level (OR=1.065) was associated with 6.5% increased odds of developing PCS. Lower levels of I-FABP (OR=0.513) suggested 48.7% reduced odds of PCS, while higher levels of sCD14 (OR=0.774) indicated a 22.6% decrease in PCS probability. No significant associations were found between serum IL-33 levels or fecal zonulin family peptide levels and the outcomes studied.

4. Discussion

Our study emphasizes the impact of SARS-CoV-2 infection on the GI which might be associated with the onset of PCS Fatigue. We propose that the viral infection dysregulates immunological pathways activating the innate immune response and the GI barrier function as indicated by the detected intestinal low-grade inflammation and a GI barrier leakage. Further, we identified pre-existing GI complaints as a risk factor for developing PCS Fatigue.

4.1 Dysregulated innate immunity may predispose for GI barrier leakage in PCS Fatigue

Elucidating mechanisms of PCS involves identifying predisposing clinical phenotypes. Recent research revealed diverse auto-antibody specificities in adaptive immunity without a clear symptom correlation [7, 8]. In our study, SARS-CoV-2 convalescent individuals have significantly higher titers of IgA (**Supplementary Figure 1**). The evaluation provides limited evidence as additional factors, such as virus variant, type of vaccination and the order of experienced immunological events, were not considered in the analyses of the SARS-CoV-2-related antibody responses and cannot be accurately assessed in our small cohort sample sizes. Early during the pandemic, low IgM or IgG3 antibodies were described to be associated with a higher risk of PCS [18]. In terms of innate immunity, the immune response, particularly changes in monocytes, dendritic cells, and mannose-binding lectin (MBL), is less understood [19, 20]. It was shown that 10 months after COVID-19, convalescent patients had lower absolute counts of granulocytes, monocytes, and lymphocytes compared to controls [10]. Low MBL levels were linked to higher cytokine levels and PCS symptoms like severe Fatigue [21]. MBL deficiency may lead to excessive IL-6 production, contributing to long-term inflammation [22]. PCS patients in our study showed significantly higher IL-6 levels compared to healthy controls (**Figure 2**). However, these results should be interpreted carefully as the assay signals in the range of the LOD. More sensitive methods are recommended for future studies. PCS patients were already susceptible to infections before SARS-CoV-2 infection (**Table 2**), indicating a possible underlying dysregulation of the innate immune system. We also observed frequent pre-existing signs of hypermobility in PCS and ME/CFS patients. Conditions like Ehlers-Danlos Syndrome and Joint Hypermobility Syndrome, often associate with systemic issues, including respiratory problems and GI complaints [23, 24]. It remains unclear which immune phenotypes may predispose for developing PCS. Genetic and epigenetic studies have previously attempted to address this question [25-27].

4.2 Multi-organ symptoms during acute COVID-19 are associated with the onset of PCS Fatigue

We were not able to observe viral shedding indicated by SARS-CoV-2 RNA in saliva, blood or stool samples in our study (data not shown). SARS-CoV-2 fecal shedding typically lasts 17.2 days on average but can continue for several months [28]. During this time, SARS-CoV-2 is not detectable over a longer period, but PCS symptoms may persist [29]. Biopsy studies in patients with GI conditions, such as IBS, IBD, or gastroesophageal reflux disease, found SARS-CoV-2 RNA or antigen in GI mucosal tissues in 50 to 70% of patients, suggesting a viral reservoir [30, 31]. However, these results came from patients with pre-existing GI diseases, leaving open whether GI damage enabled viral persistence. Pre-existing barrier or immune impairments might hinder the elimination of residual virus reservoirs or infected cells. Viral persistence, whether from SARS-CoV-2 or reactivated latent viruses like EBV, may contribute to continuous inflammation. The multi-organ spread of SARS-CoV-2 during acute infection likely contributes significantly to PCS development by disrupting immune functions, leading to prolonged immune activation or dysregulation. Our data indicate that patients with multisystemic symptoms, including GI, respiratory, and neurocognitive complaints during acute COVID-19, are more likely to develop PCS (**Supplementary Table 1**). PCS patients more often had multi-organ symptoms during acute infection compared to those who recovered. These observations align with recent literature discussed in more detail elsewhere [32, 33].

4.3 GI barrier leakage predisposes for PCS Fatigue

We showed that PCS patients had significantly more often pre-existing GI complaints before acute infection (**Table 2**). Pre-existing food intolerances, but also with a lower frequency thyroid disease, were more common in PCS and ME/CFS patients. These conditions affect the bidirectional communication between the microbiome, its metabolites, and host epithelial tissue dysregulating the gut-brain axis. In line, patients suffering from post-infectious Fatigue syndromes exhibit a dysfunctional intestinal barrier, associated with distinct fecal microbial metagenomic profiles [34]. We showed that PCS patients suffered from a significantly higher LBP/sCD14 ratio when compared to all other groups (**Figure 1**). LBP is linked to bacterial wall components that breach the intestinal barrier and enter the bloodstream [35]. The production of LBP correlates with LPS levels, as more LBP is produced as LPS concentrations increase. We did not observe major differences in sCD14 levels across the entire study cohort, even though sCD14 is required as a co-factor for LBP in mediating innate immunity [36]. Previously, it was reported that sCD14 in mice has protective effects IBD, and an increase in the LBP/sCD14 ratio correlates with elevated plasma IL-6 levels [37], which is also observable in our study (**Figure 2**). Therefore, LBP, sCD14, and their ratio are considered crucial in regulating low-grade inflammation in inflammatory diseases. We did not observe differences in I-FABP levels (**Figure 1**), which are found in elevated levels in situations of epithelial damage [38] or increased levels of fecal inflammatory marker calprotectin, β-defensin-2 and serotonin (5-HT) between all test groups (**Table 3**). Interestingly, fecal zonulin family peptides, which increase permeability in the epithelium of the small intestine by modulating the intercellular tight junctions [39], yield in a lower signal in the PCS group, but the pairwise comparisons between individual groups were not significant. Thus, no signs of severe intestinal inflammation or epithelial damage could be observed. However, it's important to note that intestinal barrier leakage occurs also in situations without an underlying damage or severe inflammation, as gastrointestinal mucosal tissues regulate their dynamics through intra- and inter-cellular transportation pathways [40]. Our findings regarding IL-1-β and IL-33 levels (**Figure 2**), which are related to gut microbiota homeostasis, support the hypothesis of mucosal barrier leakage [41, 42]. Further, we were able to show significant correlation between high LBP level and PCS.

However, our study has several limitations that should be considered. Due to the strict inclusion criteria, extensive medical records and laboratory work required, only a limited number of participants could be included. The number of ME/CFS patients is notably lower than in the other subgroups. Additionally, since the collection of anamnestic data relied mainly on open-ended questions, the self-reported data aspect provides an error risk.

5. Conclusion

Our data propose that SARS-CoV-2 disrupts pathways associated with innate immune responses and GI barrier function, leading to intestinal low-grade inflammation and mucosal barrier leakage. We identified pre-existing GI complaints as a risk factor for developing PCS Fatigue. Specific immune phenotypes, particularly in the innate immune response, appear to be critical in regulating GI barrier integrity. Our data indicate that PCS patients exhibit a higher LBP/sCD14 ratio, lower IL-33 levels and higher IL-6 levels compared to the control groups, suggesting an intestinal leakage and subsequent low-grade inflammation. Medical record data of our study cohort shows that GI complaints, but also susceptibility to infections and signs of hypermobility were already present before SARS-CoV-2 infection, in contrast to fully convalescent participants.

Overall, our study highlights the critical role of the GI tract in PCS Fatigue development. Monitoring GI symptoms and markers not only during and after an acute SARS-CoV-2 infection, but also as baseline records is needed for identifying predictive clinical phenotypes for PCS. Understanding the interplay between viral infections, immune responses, and gut integrity could lead to more effective diagnostic and treatment approaches, alleviating the burden on affected individuals and healthcare systems.

Tables

Table 1: Demographics, information on the COVID-19 disease course and vaccination state of the study cohort.

Demographics	**PCS**	**SARS-CoV-2**
Group size (n)	30	30
Mean age in years (± SD)	38 (± 9)	35 (± 12)
Sex, female	73%	70%
Mean time between SARS-CoV-2 infection and study inclusion in days (± SD)	260 (± 104)	276 (± 142)
Asymptomatic/mild COVID-19	87%	93%
SARS-CoV-2 vaccination	67%	97%
Vaccinated before first SARS-CoV-2 infection	0%	40%

Table 2 : Most prominent pre-existing complaints and comorbidities reported by study participants before their SARS-CoV-2 infection or in general for the SARS-CoV-2 negative control groups. PCS and ME/CFS patients reported a generally higher susceptibility to infections as indicated by frequently swollen lymph nodes, a sore throat and frequent respiratory infections. Pre-existing food intolerances were observed more often in PCS and ME/CFS patients. Only PCS patients had significantly more often pre-existing GI complaints. Anamnestic characteristics are presented in total numbers and percentages. Chi-squared testing was used to calculate the global p-values to determine in an explorative manner whether there is a significant difference between the study groups.

Symptoms before SARS-CoV-2 infection	**PCS**	**SARS-CoV-2, conva**
Frequent lymphadenopathy p < 0,001	**6 (20.0%)**	**0 (0.0%)**
Frequent events of sickness/infections p < 0,001	**12 (40.0%)**	**3 (10.0%)**
Frequent sore throat p < 0,001	**9 (30.0%)**	**2 (6.7%)**
Hypermobility p = 0,106	10 (33.3%)	3 (10.0%)
Any prior GI symptoms p = **0,001**	**15 (50.0%)**	**6 (20.0%)**
Diarrhea p = 0,068	4 (13.3%)	0 (0.0%)
Constipation p = **0,016**	**5 (16.7%)**	**0 (0.0%)**
Emesis p = 0,471	1 (3.3%)	0 (0.0%)
Bloating p = **0,021**	**5 (16.7%)**	**1 (3.3%)**
Abdominal pain P = 0,552	2 (6.7%)	2 (6.7%)
Stool irregularities p = 0,165	0 (0.0%)	2 (6.7%)
Reflux p = 0,176	3 (10.0%)	1 (3.3%)
Comorbidities	**PCS**	**SARS-CoV-2, conva**
Allergies (any) p = 0,758	9 (30.0%)	9 (30.0%)
Food intolerance (any) p <0,001	**13 (43.3%)**	**4 (13.3%)**
Hypercholesterolemia p = 0,717	2 (6.7%)	1 (3.3%)
Thyroid diseases p = 0,309	5 (16.7%)	1 (3.3%)
POTS p <0,001	**0 (0%)**	**0 (0.0%)**
EBV-infection confirmed by laboratory diagnostics before study inclusion p <0,001	**2 (6.7%)**	**0 (0.0%)**

Table 3: Evaluation of fecal marker associated with intestinal inflammation. Kruskal-Wallis test and Dunn's multiple comparison test were used to identify significant differences between all test groups. The Kruskal-Wallis test of fecal zonulin family peptides yields a significant p-value, but the pairwise comparisons between individual groups are not significant. No other parameter showed statistically significant difference between the groups. Global p-values are shown to determine in an explorative manner whether there is a significant difference between the study groups. LOD = Limit of detection.

Study Group	**PCS**	**SARS-CoV-2, convalescent**
Parameter (unit ± SD)		

Study Group	**PCS**	**SARS-CoV-2, convalescent**
Zonulin family peptides (ng/ml) p = 0,0323 *LOD: 0.2 ng/ml*	**218,6 (± 130,1)**	**334,2 (± 225,6)**
ἁλπροτεςτιν (μγ/μλ) p = 0,1201 *LOD: 2.3 ng/ml*	36,36 (± 65,88)	30,43 (± 29,26)
β-δεφενσιν-2 (νγ/μλ) p = 0,3746 *LOD: 15.5 pg/ml*	32,05 (± 84,52)	49,46 (± 105,1)
Σεροτονιν (5-HT, μγ/μλ) p = 0,2035 *LOD: 8.1 ng/ml*	4,811 (± 3,449)	3,519 (± 2,838)

Figure legends

Figure 1: Evaluation of serological markers indicating intestinal barrier leakage and damage. PCS patients exhibited significantly higher serum levels of Lipopolysaccharide-binding protein (LBP) compared to convalescent SARS-CoV-2 participants and healthy controls (A). In contrast, serum levels of sCD14 were significantly lower in PCS patients than in all other groups (B). When examining the LBP/sCD14 ratio, PCS patients again showed a significantly higher ratio compared to convalescent SARS-CoV-2 participants and healthy controls (C). Serum levels of I-FABP showed no differences between the groups (D). Kruskal-Wallis test and Dunn's multiple comparison test were used to identify significant differences between all test groups. * $p<0.05$. **$p<0.01$. ***$p<0.001$

Figure 2 : Evaluation of pro-inflammatory immune mediators and cytokines related to intestinal barrier integrity. Serum samples were analyzed for IFN-γ, TNF-α, IL-6, IL-1-β, IL-8, and IL-33. Significant increases in IL-6 levels and decreases in serum and IL-1-β levels were observed in PCS patients (A-C). However, many pro-inflammatory cytokine ELISA results were below the manufacturer's Limit of Detection (LOD) and had to be excluded before statistical analysis. Serum IL-33 levels were significantly lower in PCS patients compared to SARS-CoV-2 negative control participants (D). Data on IL-8 and IFN-γ levels are not shown as they did not exhibit statistical significance or a disease-specific pattern. Kruskal-Wallis test and Dunn's multiple comparison test were used to identify significant differences between all test groups. * $p<0.05$. **$p<0.01$. ***$p<0.001$

Author contributions

RJ and UE involved in conceptualization. RJ, and KL and involved in methodology. RJ, WV, SJ and ZS involved in formal analysis. SM and UE involved in resources. RJ, WV and SJ involved in data curation. RJ and UE involved in writing original draft preparation. RJ, WV, SJ, KL, SM, ZS and UE involved in writing—review and editing. RJ and UE involved in visualization. UE involved in supervision and project administration. UE involved in funding acquisition.

All authors have read and agreed to the published version of the manuscript.

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Conflict of interests

The authors declare no conflict of interest.

Institutional Review Board Statement

The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Ethics Committee of the Medical University of Vienna (vote number: 2281/2020; approved: January 19th 2021).

Informed Consent Statement

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

Data Availability Statement

The raw data supporting the conclusions of this article will be made available by the authors on request.

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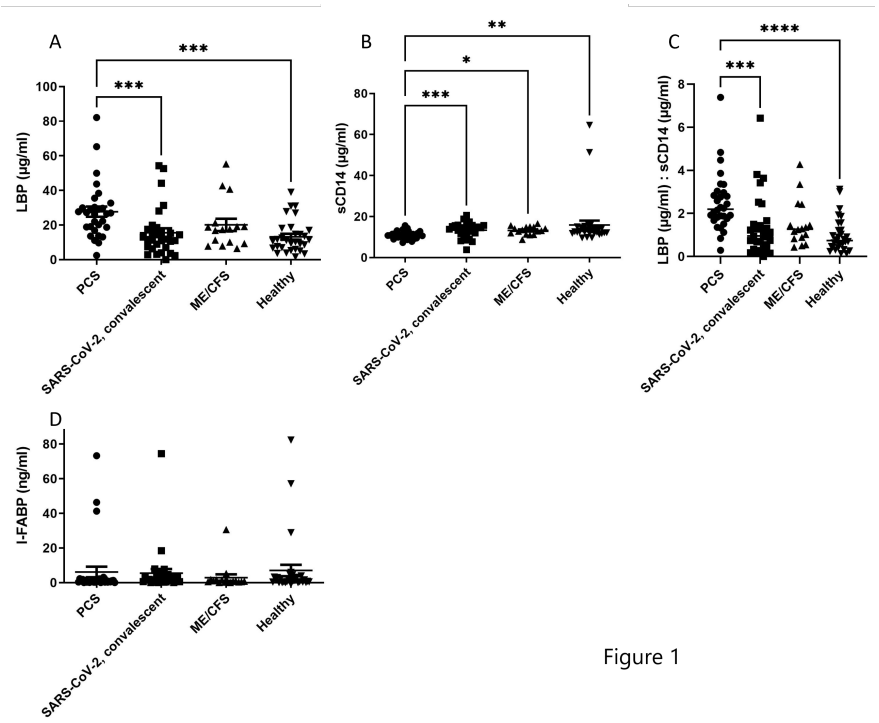

Figure 1

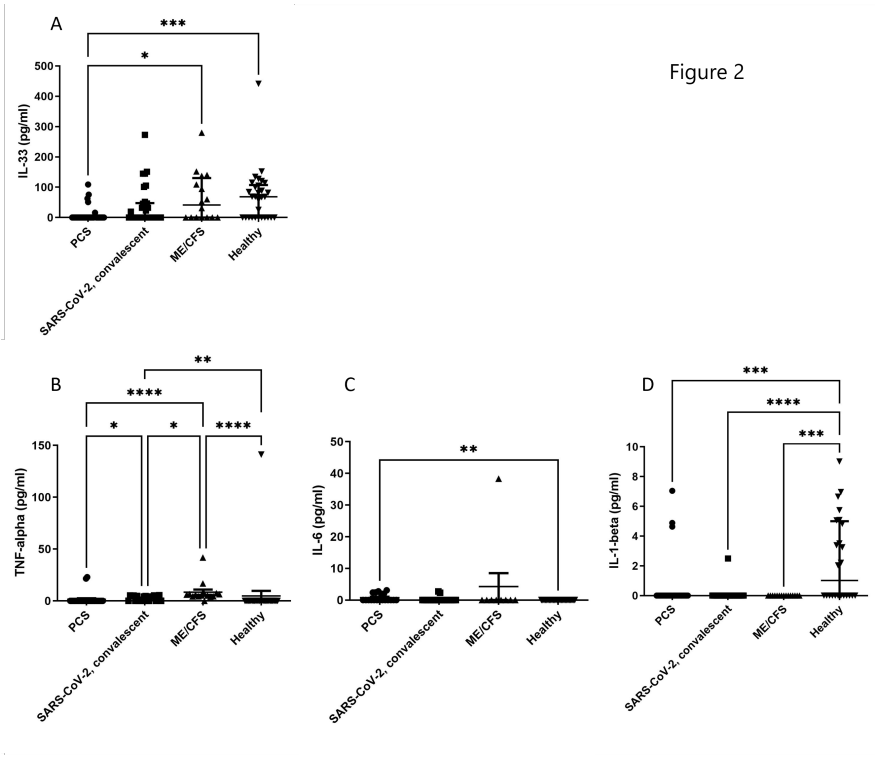

Figure 2