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Post-COVID-19 condition in prospective inpatient and outpatient cohorts

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Viral persistence, immune dysregulation, hypocortisolism, and pulmonary tissue damage from acute infection are proposed as pathogenic mechanisms underlying post-COVID-19 condition (PCC). In this prospective observational study, we followed 62 COVID-19 inpatients and 53 COVID-19 outpatients for 24 months after the infection. During this period, we assessed prolonged symptoms, lung function, and a set of immunological markers and a proportion of the patient group was assessed with computed tomography three months post-infection. The prevalence of PCC, as assessed by four medical specialists, decreased from 51% at three months to 18% at 24 months. Risk factors included the severity of the acute infection and comorbidities of obstructive sleep apnea or obesity. Patients with PCC had higher serum levels of anti-SARS-CoV-2 S1 and N protein antibodies. In the whole group, spirometry results, orthostatic hypotension, or levels of soluble suppression of tumorigenicity 2, interleukin 6 (IL-6), high-sensitivity C-reactive protein (hs-CRP), or cortisol had no association with PCC. However, using symptom clusters, patients with cognitive problems had lower cortisol levels, while patients with ongoing respiratory or myalgic symptoms had higher levels of IL-6 and hs-CRP. However, more extensive studies with clustering are needed to validate these results.

Keywords Long COVID, Viral persistence, Post-acute COVID-19 syndrome, Inflammation, IL-6, Cortisol, Viral pneumonia, Anti-SARS-CoV-2 antibodies

Meta-analyses indicate that the proportion of COVID-19 patients suffering from prolonged symptoms after acute COVID-19, referred to as post COVID-19 condition (PCC) lies between 6 and 50%[1,2]. The high variation in frequency may be caused by heterogeneity in the studies due to a bias in patient selection, the unspecific nature of the symptoms, and the lack of standardized criteria[3,4]. The identified risk factors for developing PCC include female sex, high body mass index (BMI), pre-existing pulmonary diseases (asthma or chronic obstructive pulmonary disease [COPD]), and the severity of the acute SARS-CoV-2 infection, while vaccination has a preventive effect[5–7].

Various pathophysiological mechanisms may contribute to PCC. Post-exertional malaise after COVID-19 is linked to increased mitochondrial damage, metabolic dysfunction, and capillary amyloid composition[8]. Moreover, cellular damage and the prothrombotic state caused by endothelial damage during acute SARS-CoV-2 infection can lead to organ injury and fibrosis[9]. Impairment of pulmonary function may be the result of lung damage after moderate to severe COVID-19[10,11].

SARS-CoV-2 infection can dysregulate the immune system, leading to inflammation that may last for months[12–14]. Patients with PCC have elevated serum levels of proinflammatory cytokines such as interferon- β (IFN-β), IFN-λ1, interleukin 6 (IL-6), and autoantibodies for months after acute infection[15–17]. Moreover, relief in PCC symptoms correlates with improved immune dysregulation at 24 months[18].

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A recent study showed that the soluble form of Suppression of Tumorigenicity 2 (sST2) correlates with the severity of acute COVID-19[19]. ST2 is a member of the interleukin (IL)-1 receptor family and is expressed in various cell types in exposure to stress and inflammation, while sST2 acts as a decoy receptor for IL-33[20–22]. The ST2/IL-33 pathway could be involved in PCC, as levels of sST2 are higher in patients with pulmonary embolism after COVID-19 compared to patients without previous COVID-19[23].

A few studies have found decreased cortisol levels among patients with PCC, suggesting that the hypothalamic-pituitary-adrenal axis may be involved in the disease pathogenesis[24,25]. However, the studies were criticized for not accounting for circadian and ultradian oscillations in physiological cortisol production, and some other studies have not been able to replicate the findings[26,27].

Viral persistence could explain prolonged inflammation after acute infection and prolonged symptoms. Indeed, there is growing evidence for the persistence of viral RNA and proteins in different tissues associated with PCC[28–33]. Normal antibody response against SARS-CoV-2 peaks approximately 1–2 months post-infection, but PCC patients have persistently elevated levels of antibodies against SARS-CoV-2 spike (S) glycoprotein and nucleocapsid (N) protein[24,34,35].

Some studies have clustered the patients according to the most dominant symptoms to differentiate between potential mechanisms, such as lung damage after severe COVID-19, symptoms related to persistent inflammation, and brain involvement. The most common individual clusters are cardiorespiratory, systemic inflammatory, and neurocognitive[36,37].

In this prospective 24-month study, we explored the role of inflammation, viral persistence, and hypocortisolism in PCC in a combined cohort of inpatients and outpatients followed after acute COVID-19 infection. Clinical data with patient history and self-reported symptoms were combined to diagnose PCC. We evaluated the levels of serum sST2, IL-6, high-sensitivity CRP (hs-CRP), cortisol, and antibodies against SARS-CoV-2 S1 and N proteins between PCC patients and recovered individuals. Finally, we compared the inflammatory and humoral responses in three different PCC clusters.

Methods

Study design and patients

This was a prospective cohort study with a two-year follow-up (Fig. 1). The study is a sub-study of a Clin-COVID master study[38]. We enrolled 62 inpatients and 53 COVID-19 outpatients in the acute phase of infection during two waves of infections from March 21 to December 13, 2021. Patients were enrolled in two one-month periods, in March and December. We aimed to have different age groups represented, and outpatients were recruited simultaneously with the inpatients. The inclusion criteria were PCR-confirmed SARS-CoV-2 infection and age over 18 years. Inpatients were recruited to the study on the second day of admission to Turku University Central Hospital (Tyks), while outpatients were contacted by telephone after the positive COVID-19 test result and invited to the research clinic for enrollment after the two-week isolation period. Patients were examined by a study physician (AH, AV) at three months and six months, followed by an interview by telephone call (AH) at 12 months and 24 months.

Surveys and clinical data

No validated questionnaire was available to assess PCC symptoms during the study. Therefore, we designed a questionnaire based on available evidence and clinical experience to evaluate the symptoms and detailed medical history (supplementary information). This online questionnaire was filled three months, six months, one year,

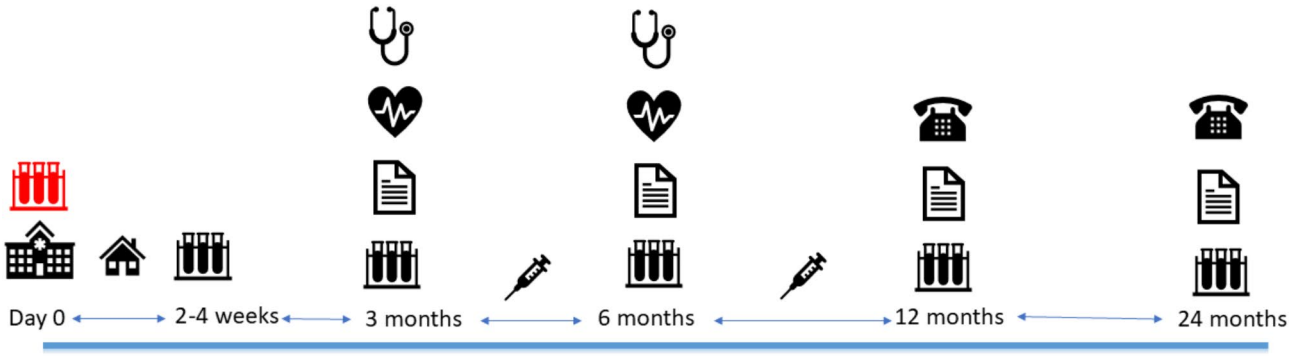

Fig. 1. Study design. Inpatients (black hospital pictogram) were recruited on the second day of admission, while outpatients (black house pictogram) were recruited after a two-week isolation period. Afterward, the two groups were assessed with the same methodology. Acute phase samples were collected during the hospitalization (red sample pictogram), and the rest of the serum samples were collected one, three, six, twelve, and 24 months post-infection (black sample pictogram). Patients were examined by study physicians (black stethoscope pictogram) and study nurses (black heart pictogram) three and six months post-infection. Study physicians interviewed the patients by phone at 12 months and 24 months (black telephone pictogram). The study questionnaire was filled out three, six, twelve, and 24 months post-infection (black sheet pictogram). Most patients received COVID vaccinations (adenoviral vector or mRNA-based) between three and 12 months post-infection (black syringe pictogram).

and two years after the acute infection. The answers were assessed using a numerical rating scale from 0 to 10 on the questions with verbal descriptors. At three months post-infection, patients were also asked retrospectively to score the severity of symptoms before and during COVID-19. For statistical analysis, an increase of at least four points on the numerical rating scale compared to the pre-infection situation was defined as a potential PCC symptom. Participants self-reported their use of alcohol or nicotine products and COVID-19-related absence from work.

We used questionnaire and patient record data for the definition of acute disease severity, including acute symptoms and the mode of respiratory support among inpatients (supplemental oxygen, non-invasive ventilatory support, and mechanical ventilation). Data on demographics, comorbidities, and medications were collected during patient visits and from patient records. The severity of the acute disease was categorized on a scale from one to four according to symptoms and the need for medical support (supplementary information).

We evaluated the pulmonary function at three and six months post-infection using spirometry and a six-minute walking test (6MWT) according to the European Respiratory Society (ERS) guidelines[39]. Spirometry was performed using a Medikro Pro™ spirometer (Medikro, Kuopio, Finland) under the supervision of a trained study nurse. The values were compared to age-, sex-, and height-adjusted reference values[40].

Cardiovascular function was assessed with electrocardiography (ECG) and an orthostatic test at three and six months. At three months, high-resolution computed tomography (HRCT) of the lungs was taken on clinical grounds from 24/38 (63%) inpatients and 27/48 (56%) outpatients. In 4/38 (11%) inpatients and 1/48 (2%) outpatients, HRCT was repeated for clinical indication at the six-month follow-up.

Diagnosis and clustering of post-COVID-19 condition (PCC)

The diagnosis of PCC was determined by four study physicians independently (AH, AV, JO, TF) by evaluating the severity of symptoms and possible explanation by other clinical conditions, following the WHO case definition[2]. In case of discordance between the experts, the cases were discussed to reach an agreement on the possible existence and categorization of PCC.

PCC patients were manually distributed into three symptom clusters corresponding to their most dominant self-reported symptoms according to Global Burden of Disease Long COVID Collaborators guidelines[37] (supplementary information). Shortly, persistent fatigue with myalgia or mood swings (cluster 1) included bodily pain (myalgia), weakened muscular strength, numbness and tingling, fatigue, and mood disturbances. Cognitive problems (cluster 2) included attention deficit and cognitive difficulties, and ongoing respiratory problems (cluster 3) included exertional and resting dyspnea, persistent cough, and thoracic pain.

Sampling

Diagnosis of COVID-19 at the time of enrollment was based on positive SARS-CoV-2 RT-PCR results from the nasopharyngeal (NP) swabs taken at Tyks Emergency Clinic or Turku Health Centre COVID-19 testing facilities. The medical staff of the local healthcare providers examined all suspected cases and took NP swabs. Serum samples were collected at one month, three months, six months, 12 months, and 24 months after the infection. For inpatients, additional acute-phase serum samples taken during the hospitalization were included in the analysis. All serum samples were stored at -80 °C until used in the analyses.

Detection of anti-SARS-CoV-2 S1 and N protein antibodies

SARS-CoV-2 S1 and N-specific antibodies were measured with an enzyme immunoassay (EIA) as described previously with slight modifications[41]. Briefly, 96-well plates were coated with purified recombinant S1 subunit and N proteins (3.5 µg/ml S1 and 2.0 µg/ml N) and blocked with assay buffer (5% swine serum and 0.5% Tween-20 in PBS). Serum samples were diluted 1:1000 for anti-S1 IgG EIA and 1:300 for all other EIAs and serum samples were incubated for two hours at + 37 °C. After washing, anti-human IgG (1:8000 dilution), IgA (1:8000), or IgM (1:4000) antibodies conjugated to HRP (Dako, Agilent, USA) were added and incubated for one hour at + 37 °C. All different antibody subclasses were analyzed separately, each in their respective assay. The amount of bound primary antibodies and secondary HRP conjugate was detected with TMB One substrate (100 µl/well), and the reaction was measured at 450 nm after stopping with sulfuric acid (0.2 N; 100 µl/well). Linear interpolation between the optical density (OD) values of the positive (100 units) and negative control (0 units) serum samples was used to convert the OD values to EIA units. Cut-off values for seropositivity were determined as an average plus three times the standard deviation of 52 serum samples collected from uninfected COVID-19-vaccinated individuals before the first vaccine dose[41].

Detection of serum sST2 and IL-6

Serum sST2 and IL-6 levels were measured with a Human sST2 enzyme-linked immunosorbent assay (ELISA) kit (Elabscience Biotechnology, Wuhan, China) and IL-6 Human ELISA kit (BioVendor, Brno, Czech Republic) according to the instructions of the manufacturer. All collected samples from the same patient were analyzed on the same plate to avoid possible interassay variation. The detection ranges sST2 and IL-6 were 0.31–20 ng/mL and 1.25–80pg/mL, respectively. The sST2 and IL-6 kits had a sensitivity of 0.19 ng/mL and 0.65pg/mL, respectively. Both kits had intra- and inter-kit coefficients of variation of less than 10%. The values below the limit of detection (LOD) were substituted with half of the lowest LOD.

Detection of serum hs-CRP and cortisol

Plasma cortisol levels were analyzed by electrochemiluminescence assay (Cobas e801, Roche Diagnostics, Mannheim, Germany) and serum hs-CRP using nephelometry assay (BN ProSpec, Siemens Healthineers AG, Munich) at the Department of Clinical Chemistry, Turku University Hospital, according to the standard protocols.

Statistical analysis

Study data were collected and managed using REDCap electronic data capture tools hosted at Turku University. Continuous variables were summarized with median and interquartile range (lower quartile-upper quartile) due to skewness of data distributions. Categorical variables are reported with counts and percentages. Association between two categorical variables were studied with Fisher's exact test or chi-square test. Associations of explanatory variables with PCC were assessed in age- and sex-adjusted logistic regression models and reported in odds ratios (OR) with 95% confidence intervals (CI). To study the behavior of anti-SARS-CoV-2 S1-specific antibody responses over time, we built up a linear mixed model suitable for repeated measurements. Model included gender, age, PCC status at three months, time (within-factor), and PCC by time interaction. Within this model, we also estimated mean differences at each time point. Compound symmetry covariance structure was used, as well as Kenward-Roger correction for degrees of freedom. Natural logarithmic or square root transformations were used for many variables due to the skewness of the distributions. Normality assumptions were checked with studentized residuals. The data analysis for this paper was generated using SAS software, Version 9.4 of the SAS System for Windows (SAS Institute Inc., Cary, NC, USA). GraphPad Prism version 10.0 was used for a four-parameter logistic (4PL) curve model to analyze EIA data.

Results

The median age of the inpatients was 52.5 years (95% confidence interval [CI] 49.9–56.0 years), with 44% (27/62) female, while the median age of the outpatients was 33.3 years (95% CI 30.7–35.9 years), with 62% (33/53) female (Table 1). Inpatients had higher BMI and more comorbidities, notably asthma, obstructive sleep apnea, and type 2 diabetes than outpatients. The predominant variant in Finland from March 13 to June 6, 2021, was Alpha, and from November 11 to December 13, 2021, Delta[42,43].

The prevalence of PCC was 51% (43/84) at three months, 30% (22/74) at six months, 23% (16/70) at one year, and 18% (11/60) at two years post-infection (Table 2). Figure 2 presents the patients with the patient flow during the study. Fatigue was the most reported PCC-associated symptom at every follow-up. The frequencies of all symptoms are shown in (Supplementary Table 1).

As risk factors for PCC at three months, we identified higher BMI (OR 1.12 [95% CI 1.03–1.22], $p = 0.007$) and obstructive sleep apnea (OR 5.16 [1.04–25.6], $p = 0.04$) (Table 3). The severity of acute disease correlated

Category	All	Inpatients	Outpatients	PCC (3 months)	nPCC (3 months)
n (%)	115	62 (53,9%)	53 (46,1%)	43 (51.2%)	41 (48.8%)
Age in years, median (95% CI)	42.7 (39.8–45.6)	52.5 (49.0–56.0)	33.3 (30.7–35.9)	43.4 (39.3–47.6)	41.2 (36.2–46.2)
Female sex	52.2%	43.3%	62.3%	51.2%	58.5%
BMI (kg/m^2)	29.7	32.0	27.2	32.0	27.4
Hospitalized, n (%)	62 (53,9%)	NA	NA	27 (62,8%)	15 (36,6%)
Home-treated, n (%)	53 (46,1%)	NA	NA	16 (37,2%)	26 (63,4%)
Current or prior smoker	40.7%	37.3%	45.8%	46.5%	39.0%
At least one comorbidity	64.3%	90.5%	47.6%	53.5%	46.3%
≥ 2 comorbidities	34.5%	52.4%	35.7%	25.6%	19.5%
Asthma or COPD	20.2%	33.3%	0.0%	20.9%	14.6%
Obstructive sleep apnea	15.5%	28.6%	0.0%	20.9%	4.9%
Hypertension	25.0%	45.2%	47.6%	16.3%	19.5%
Type 2 Diabetes	10.7%	14.3%	7.1%	7.0%	7.3%
Anxiety or depression	14.3%	14.3%	14.3%	9.3%	9.8%
Severity of acute disease					
1 = Very mild	34.0%	0.0%	73.5%	20.9%	51.2%
2 = Mild	20.8%	20.0%	26.5%	27.9%	19.5%
3 = Moderate	24.5%	43.3%	0.0%	32.6%	19.5%
4 = Severe	20.8%	36.7%	0.0%	18.6%	9.8%
Antibiotics during acute disease	34.9%	65.0%	2.0%	34.9%	26.8%
Antibiotics after acute disease	4.7%	5.0%	4.1%	7.0%	4.9%
HRCT					
Abnormal CT	41.2%	61.5%	20.0%	45.5%	33.3%
Mosaic perfusion	17.6%	19.2%	16.0%	18.2%	16.7%
Ground glass opacity	35.3%	57.7%	12.0%	42.4%	22.2%
Fibrosis	3.9%	7.7%	0.0%	3.0%	5.6%
post-COVID changes*	25.5%	46.2%	4.0%	33.3%	11.1%

Table 1. General characteristics of patients. *BMI* body mass index, *CI* confidence interval, *COPD* chronic obstructive pulmonary disease, *CT* computed tomography, *nPCC* non-post-COVID-19 condition patients, *PCC* post-COVID-19 condition patients, *NA* not assessed, * as determined by a thoracic radiologist.

	3 months		6 months		12 months		24 months	
	nPCC	PCC	nPCC	PCC	nPCC	PCC	nPCC	PCC
n (%)	41 (48.8%)	43 (51.2%)	52 (70.3%)	22 (29.7%)	54 (77.1%)	16 (22.9%)	49 (81.7%)	11 (18.3%)
Age in years, median	41.2	43.4	40.5	43.9	41.9	45.0	42.5	46.4
BMI	27.3	32.4	28.1	32.3	28.8	32.9	29.1	34.0
Female sex	58.5%	51.2%	57.7%	45.5%	59.3%	47.1%	63.3%	36.4%
Treatment center								
Hospitalized, n (%)	15 (36.6%)	27 (62.8%)	21 (40.4%)	14 (63.3%)	24 (44.4%)	9 (56.2%)	23 (47.9%)	8 (72.7%)
Home treated, n (%)	26 (63.4%)	16 (37.2%)	31 (59.4%)	8 (36.4%)	30 (55.6%)	7 (43.8%)	25 (52.1%)	3 (27.3%)
Severity of the acute disease								
1 = Very mild, n (%)	21 (51.2%)	9 (20.9%)	22 (42.3%)	7 (31.8%)	21 (38.9%)	6 (35.3%)	19 (38.8%)	3 (27.3%)
2 = Mild, n (%)	8 (19.5%)	12 (27.9%)	13 (25.0%)	4 (18.2%)	14 (25.9%)	2 (11.8%)	13 (26.5%)	1 (9.1%)
3 = Moderate, n (%)	8 (19.5%)	14 (32.6%)	13 (25.0%)	5 (22.7%)	14 (25.9%)	4 (23.5%)	13 (26.5%)	3 (27.3%)
4 = Severe, n (%)	4 (9.8%)	8 (18.6%)	4 (7.7%)	6 (27.3%)	5 (9.3%)	5 (29.4%)	4 (8.2%)	4 (36.4%)
Physiological tests								
Severe orthostatism, n (%)	2 (4.8%)	1 (2.4%)	5 (9.8%)	1 (4.5%)	NA	NA	NA	NA
Moderate orthostatism, n (%)	5 (11.9%)	4 (9.8%)	6 (11.8%)	5 (22.7%)	NA	NA	NA	NA
Walking distance (%) in 6MWT, median (IQR)	95.5 (88.0–104.0)	96.0 (87.0–107.0)	99.9 (90.5–108.7)	94.4 (90.4–113.3)	NA	NA	NA	NA
FVC (Z-value), mean ± 95% CI	−0.1 (−0.34–0.14)	−0.7 (−1.14–[−0.26])	−0.6 (−0.97–[−0.23])	−0.7 (−1.17–[−0.23])	NA	NA	NA	NA
FEV1 (Z-value), mean ± 95% CI	−0.1 (−0.33–0.13)	−0.5 (−0.87–[−0.13])	−0.7 (−1.06–[−0.34])	−0.8 (−1.28–[−0.32])	NA	NA	NA	NA
PEF (Z-value), mean ± 95% CI	−1.1 (−1.32–[−0.88])	−1.2 (−1.60–[−0.80])	−1.1 (−1.43–[−0.77])	−0.8 (−1.30–[−0.30])	NA	NA	NA	NA

Table 2. Baseline characteristics of patients with and without PCC. *6MWT* six-minute walking test, *BMI* body mass index, *FEV1* forced expiratory volume in one second, *FVC* forced vital capacity, *PEF* peak expiratory flow, *NA* not assessed.

with the prevalence of PCC (Fisher's Exact Test, $p = 0.04$), but the difference was not statistically significant when corrected for sex and BMI ($p = 0.28$). Although ground glass opacity and radiological post-COVID changes were more prominent in the PCC group, the findings in HRCT imaging did not reveal any statistically significant differences between the groups (Table 1). We also found no significant differences in the 6MWT or spirometry results between the PCC and nPCC groups (Table 2 and Supplementary Fig. 1). However, a subgroup of PCC patients experiencing pulmonary symptoms (cluster 3) had more restrictive and obstructive spirometry results than the other two clusters (Supplementary Table 3).

Patients with PCC had more often moderate orthostatism at six months, but the difference was not statistically significant (22 vs. 11%, $p = 0.4$). Moreover, patients with PCC and recovered individuals had no significant differences in 6MWT or spirometry at three- or six-month follow-ups (Table 2 and Supplementary Fig. 1). The most common PCC cluster among inpatients was ongoing respiratory problems (cluster 3; 52% [14/27])) (Table 4). Among outpatients, none of the clusters was dominant.

Specific antibodies in PCC and non-PCC patients

The median levels of anti-S1 and anti-N IgG antibodies did not significantly differ throughout the follow-up period, nor did they predict the risk of PCC, except for anti-N IgG at three months post-infection (OR 1.019, $p = 0.048$) (Fig. 3, Supplementary Table 2). After excluding participants with recent vaccinations at six and twelve months, the odds ratios of anti-S1 and anti-N IgG antibody levels explaining PCC increased but remained statistically insignificant (Supplementary Table 2). A significant increase in anti-S1 antibody levels at 6 months was associated with recent vaccination in concordance with national guidelines at the time (data not shown).

PCC patients had more variation in median anti-N IgA and IgM antibody levels among the inpatient and outpatient cohorts (Supplementary Fig. 3C, D), but the overall median levels did not differ significantly (Supplementary Fig. 4).

Inflammation markers in PCC and non-PCC patients

For all patients, the median levels of sST2 were higher at the acute phase at 21.3 ng/ml and then declined to a steady level of 12.7–14.0 ng/ml. The median level of IL-6 was also highest at the acute phase at 10.6 pg/ml and then declined to 4.5–5.4 pg/ml, peaking once at 6.1 pg/ml at six months post-infection. The median level of hs-CRP was highest at the acute phase at 20.3 mg/l and then declined to a steady level of 1.6–2.2 mg/l. The median cortisol levels were also highest at the acute phase at 428 nmol/l and steadily declined to 279 nmol/l at 24 months post-infection.

During the acute phase, PCC patients had significantly higher median levels of proinflammatory proteins sST2 (28.2 vs. 20.9 ng/ml, $p = 0.031$) and hs-CRP (39.2 vs. 2.9 ng/l, $p = 0.002$), and significantly lower cortisol

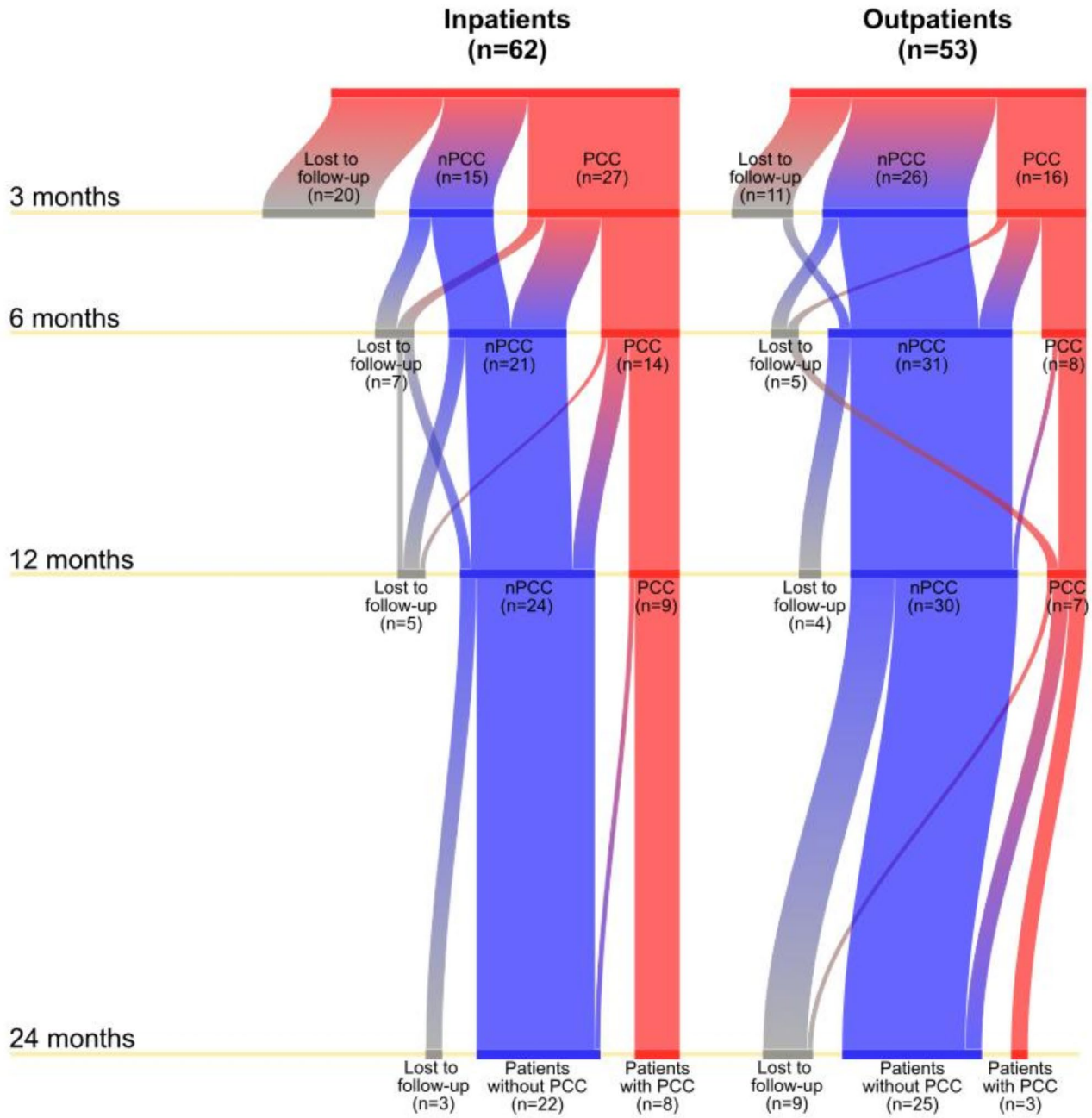

Fig. 2. Patient flow in the study. *nPCC* non-post-COVID-19 patients, *PCC* post-COVID-19 patients.

levels as compared to recovered patients (307 vs. 456 nmol/l, $p = 0.005$) (Fig. 4). Inpatients with PCC had lower cortisol levels in the acute phase, but the difference was insignificant for outpatients (Supplementary Fig. 5C and D).

The IL-6 levels appeared slightly higher in the PCC group at three, six, and twelve months post-infection, but the differences were statistically insignificant (Fig. 4 and Supplementary Table 2). The differences were concordant among inpatient and outpatient cohorts but remained statistically insignificant (Supplementary Fig. 6A and B).

Otherwise, no significant differences existed in any of the measured parameters between PCC patients and recovered individuals from six months onward. Correcting for recent vaccination did not change the results. At the two-year follow-up, hs-CRP levels were low in both groups but marginally higher in the PCC group, with an almost significant difference (2.6 vs. 1.3 mg/l, $p = 0.07$). However, among the inpatient or outpatient cohorts, we found no concordant differences in hs-CRP levels (Supplementary Fig. 5C and D).

	3 months		6 months		12 months		24 months	
Risk factor	**OR**	**p**	**OR**	**p**	**OR**	**p**	**OR**	**p**
BMI	1.119 (1.031–1.215)	0.007	1.059 (0.976–1.149)	0.17	1.073 (0.986–1.167)	0.10	1.057 (0.966–1.156)	0.23
Sex (male)	0.257 (0.227–0.634)	0.63	1.280 (0.373–4.394)	0.70	0.332 (0.445–0.505)	0.51	0.310 (0.383–0.658)	0.42
Obstructive sleep apnea	5.161 (1.042–25.556)	0.04	2.296 (0.461–11.438)	0.31	1.536 (0.344–6.858)	0.57	1.828 (0.396–8.438)	0.44
Asthma or COPD	0.679 (0.231–1.997)	0.48	0.776 (0.184–3.276)	0.73	1.427 (0.273–7.460)	0.67	1.066 (0.196–5.809)	0.94
Diabetes	3.000 (0.299–30.079)	0.35	2.526 (0.387–16.506)	0.33	2.410 (0.364–15.981)	0.36	1.467 (0.138–15.614)	0.75
Hypertension	NA	0.15						
Anxiety	NA	0.51						
Depression	NA	0.32						

Table 3. Risk factors associated with post-COVID-19 condition (PCC). The association of PCC with body mass index, sex, obstructive sleep apnea, chronic obstructive pulmonary disease or asthma, and diabetes was analyzed using logistic regression. The correlation between hypertension, anxiety, and depression with PCC was analyzed with Fisher's exact test or chi-squared test. *OR* odds ratio, *NA* not assessed.

	3 months		6 months		12 months		24 months	
	Inpatients	**Outpatients**	**Inpatients**	**Outpatients**	**Inpatients**	**Outpatients**	**Inpatients**	**Outpatients**
PCC cluster								
Fatigue with myalgia (cluster 1), n (%)	7 (25.9%)	6 (37.4%)	5 (35.7%)	3 (37.5%)	4 (44.4%)	2 (28.6%)	5 (62.5%)	1 (33.3%)
Cognitive problems (cluster 2), n (%)	6 (22.2%)	5 (31.3%)	1 (7.1%)	3 (37.5%)	1 (11.1%)	2 (28.6%)	1 (12.5%)	2 (66.6%)
Ongoing respiratory problems (cluster 3), n (%)	14 (51.9%)	5 (31.3%)	8 (57.1%)	2 (25.0%)	4 (44.4%)	3 (42.8%)	2 (25.0%)	0 (0.0%)

Table 4. Clusters of PCC during the study in among inpatient and outpatient cohorts.

Acute disease severity

The acute phase levels of sST2, IL-6, and hs-CRP were significantly higher, and cortisol levels were lower in patients with severe disease than those with mild acute disease (Supplementary Fig. 7A–D). After six months, the sST2 levels returned to low levels in all patients. Patients with a severe acute disease had higher levels of anti-N IgA at the acute phase and anti-S1 IgG and anti-S1 IgM antibodies for up to three months post-infection compared to patients with a mild disease (Supplementary Fig. 8A, C, E, and F). Afterward, the difference disappeared, possibly with patients with mild and moderate diseases getting vaccinations. However, the differences in anti-N IgG and IgM antibody levels in patients with a severe acute disease also disappeared after six months (Supplementary Fig. 8B and D).

PCC symptom clustering

Cluster 3 patients, with persistent respiratory symptoms, were often female and had higher BMI, while cluster 2 patients, with cognitive symptoms, were younger. Cluster 3 patients had more restrictive and obstructive spirometry results than those in the other two clusters, but there were no differences in 6MWT or HRCT findings (Supplementary Table 3). We used a mixed linear model to evaluate median level differences in the parameters among the clusters. We found that clusters 1 and 3 had similar median levels in many variables compared to cluster 2, which was a more separate group from the others, although the differences were not statistically significant.

At three months, clusters 1 and 3 had higher levels of anti-S1 IgG, but the difference was not statistically significant (33.0 vs. 10.6 EIA units, $p = 0.14$, and 43.3 vs. 10.6 EIA units, $p = 0.14$). At six months, the differences in anti-S1 IgG between clusters 1 and 2 and clusters 3 and 2 were significant (101.3 vs. 54.5 EIA units, $p = 0.0122$, and 91.3 vs. 54.5 EIA units, $p = 0.0118$, respectively). However, after six months, the differences between the groups were not statistically significant, likely due to the diminishing sizes of the subgroups (Supplementary Fig. 9A and B). The clusters had no differences in anti-S1 IgM and IgA or anti-N IgG antibody levels. The differences in hs-CRP levels were subtle but consistent and statistically significant at six months between clusters 1 and 2 (2.0 vs. 0.7 mg/l, $p = 0.017$) and clusters 3 and 2 (2.8 vs. 0.7 mg/l, $p = 0.015$) and at 24 months between clusters 1 and 2 (3.0 vs. 0.6 mg/l, $p = 0.018$), and clusters 3 and 2 (10.7 vs. 0.6 mg/l, $p = 0.017$) (Supplementary Fig. 10D).

During the 24-month follow-up, cluster 2 patients had lower cortisol levels than those belonging to clusters 1 and 3 (Supplementary Fig. 10 C). Twelve months post-infection, the difference was statistically significant (179 vs. 290 nmol/l, $p = 0.031$, and 179 vs. 351 nmol/l, $p = 0.054$). However, while the trend was similar throughout the follow-up, the differences did not reach statistical significance at any other time point, likely due to the small subgroup sizes.

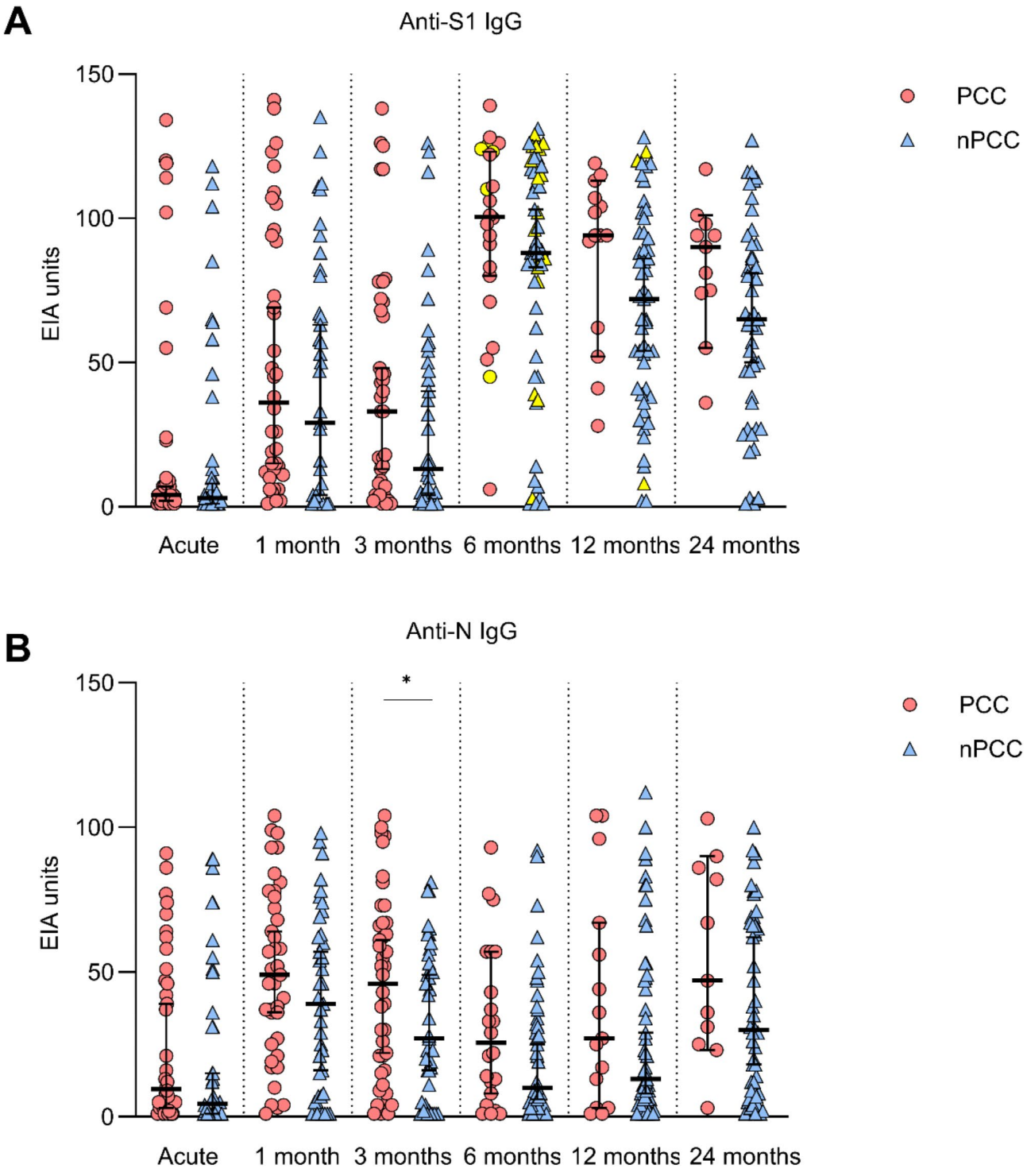

Fig. 3. Humoral IgG responses in patients with PCC and recovered individuals (nPCC). (**A**) Anti-SARS-CoV-2 S1-specific IgG antibody responses in samples collected at each time point. Yellow dots represent samples from patients vaccinated less than a month before sampling. (**B**) Anti-SARS-CoV-2 N-specific IgG antibody responses. The evaluation of PCC at the acute phase and a month post-infection was based on the evaluation at the three-month follow-up. Otherwise, PCC status was evaluated at the corresponding follow-up. The black lines represent median levels with a 95% CI of the median level. The antibody levels at each time point were compared with linear mixed models suitable for repeated measurements adjusted with sex and age. $^{*}p < 0.05$.

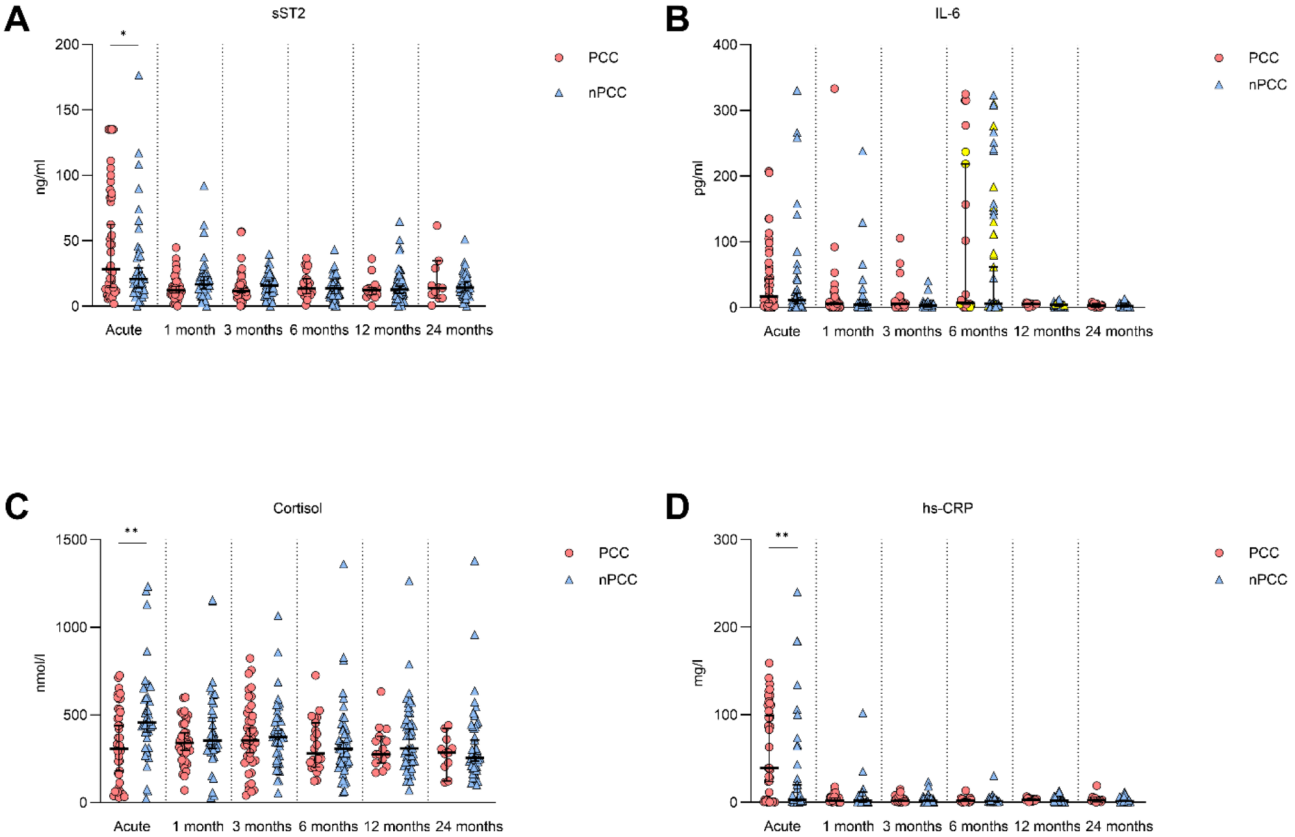

Fig. 4. Serum levels of sST2, IL-6, cortisol, and hs-CRP in patients with PCC and recovered individuals (nPCC). (**A**) Levels of sST2 in samples collected at each time point. (**B**) Levels of IL-6 at each time point. Yellow dots represent samples from patients vaccinated less than a month before sampling. (**C**) Levels of cortisol at each time point. (**D**) Levels of hs-CRP at each time point. The evaluation of PCC at the acute phase and a month post-infection was based on the evaluation at the three-month follow-up. Otherwise, PCC status was evaluated at the corresponding follow-up. The black lines represent median levels with a 95% CI of the median level. The levels of each parameter were compared with linear mixed models for repeated measurements adjusted with sex and age. $*p < 0.05$; $**p < 0.01$.

Discussion

Our study is one of the few to investigate the role of PCC with the same set of variables and immune markers in inpatients and outpatients. We avoided selecting patients based on their self-reported symptoms to minimize bias toward recruiting predominantly patients with long-term sequelae. We found that the relatively high proportion of patients with PCC reduced from 51% at three months to 18% at two years post-infection. However, as the diagnosis of PCC was made using self-reported symptoms, we had to arbitrarily select a threshold of four or more in the numerical rating scale for a positive symptom. This could affect the prevalence of PCC, and this needs to be considered when comparing these results to those of other studies. Additionally, at three months, some patients could still experience some symptoms from the acute infection, explaining the high prevalence of PCC.

We observed elevated acute phase levels of sST2 and hs-CRP in PCC patients diagnosed at three months. This could be due to these patients having a more severe form of acute COVID, which has been shown to induce significant production of these markers[19,44]. The overall cortisol levels were significantly lower in the acute phase – especially among inpatients with a severe disease. This can result from treatment with corticosteroids, suppressing endogenous cortisol production. However, we did not collect the samples uniformly at the same time of the day, resulting in increased variation in cortisol levels. Therefore, these results need to be carefully interpreted. PCC was not associated with differing levels of IL-6, HRCT findings, exercise capacity (6MWT), or lung function. However, cluster 3 patients with ongoing respiratory problems had reduced lung function with restrictive and obstructive features in spirometry. This indicates that the severity of the acute illness correlates with PCC and that prolonged respiratory symptoms can be related to spirometry findings, possibly due to lung tissue damage. Lung damage has been reported in inpatients after severe COVID-19 infection, but we found post-COVID changes also in some outpatients[10].

We found that patients with PCC seemed to have increased serum levels of anti-S1 and anti-N antibodies, but the difference was statistically not significant. However, the visual trend was concordant among the inpatients and outpatients (Supplementary Fig. 2A, B, 3 A, B) and this trend was seen throughout the follow-up among inpatients and outpatients. This could be explained by viral persistence causing prolonged inflammation through

constant immune activation[32,45,46]. Higher levels of anti-SARS antibodies have been reported in patients with inadequate viral clearance[47]. The serum levels of antiviral IgA and IgM antibodies were low in both PCC and non-PCC groups, likely due to the relatively rapid decay of these antibody classes[48].

The identification of different symptom clusters offered valuable insights. Patients exhibiting fatigue with myalgia and patients with ongoing respiratory problems had elevated levels of anti-S1 antibodies, hs-CRP, and IL6, while patients with cognitive problems had decreased cortisol levels. Fatigue and myalgia, prominent symptoms in cluster 1, could be associated with persistent inflammation[49]. Cognitive disturbances in cluster 2 patients may result from impairment in the blood-brain barrier functions[50] or functional neurological disorders[51]. However, based on our research, hypocortisolism could be a pathophysiological mechanism in this population, but additional research is needed to verify this finding. Dyspnea, instead, prevalent in cluster 3, may be linked to pulmonary damage[10].

Our study had limitations due to the diverse nature of the PCC and our small-sized cohort. Thus, the subgroups were too small for reliable statistical comparisons. Additionally, our study design relied on a retrospective collection of data for the acute phase symptoms of COVID-19 and pre-existing symptoms, which may have created biases or inaccuracies in the reported symptoms or overdiagnosis of PCC. The lack of internationally standardized criteria for PCC may have further contributed to variability in the diagnosis and characterization of the condition. Moreover, we did not include an age and gender-matched negative control group to assess the psychosocial effect of the pandemic on the perceived symptoms[52]. Our previous study comparing outpatients recruited during acute respiratory tract infection found that PCC-associated symptoms were also common in the PCR-negative outpatient cohort; the only symptoms more prevalent in the PCR-positive outpatient cohort were anosmia and ageusia[4]. However, the comparison between PCR-positive inpatients and outpatients revealed higher rates for inpatients, consistent with the findings of the present study.

Conclusions

Overall, our study showed that a severe COVID-19 predisposes to persistent symptoms after infection. Still, similar symptoms were also found with lower frequency among outpatients who suffered from a mild to moderate form of COVID-19. A rewarding feature was that in most patients, the PCC symptoms disappeared within 1 to 2 years of the follow-up. Inflammatory markers, such as sST2 and CRP, were elevated, and cortisol levels were reduced in serum specimens at the acute phase of the disease, especially in severely infected individuals. The acute phase levels of sST2, IL-6, and hs-CRP decreased more in the PCC group, likely reflecting appropriate treatment of inflammation during hospitalization. These markers returned to low levels within three to six months, and no difference was seen between prolonged PCC and recovered patients in general. Thus, their analyses may not provide a reliable prognostic factor of PCC disease activity. However, for subgroups of PCC, we noticed small differences in proinflammatory proteins and cortisol, possibly due to differencies in pathophysiology. In the future, cohort studies with stricter criteria for classifying patients into specific clusters are needed to understand better the role of inflammation in PCC and other post-viral syndromes.

Data availability

The datasets generated and analyzed during the current study are available from the corresponding author upon reasonable request.

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Author contributions

A.H., A.V., J.O., Q.H., T.V., T.F., and I.J designed the study. A.H. and A.V. examined the patients. A.H., A.V., T.F., and J.O. diagnosed the post-COVID-19 conditions and clustered the patients. A.H., P.J., and J.T. designed and performed the experiments. A.H., P.J., J.T., and E.L. analyzed the data. T.V., A.K., and J.O. contributed to the data

collection and design. A.H., Q.H. and I.J. wrote the manuscript, and all authors revised and approved the manuscript for publication. All authors contributed to the article and approved the submitted version.

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Declarations

Competing interests
The authors declare no competing interests.

Ethics
The study protocol was approved by the Helsinki-Uusimaa Hospital District (HUS) Regional Committee on Medical Research Ethics (permission number HUS/1238/2020). The study adheres to the principles of the Declaration of Helsinki and is conducted according to the principles of Good Clinical Practice. All participants provided written informed consent upon recruitment to the study.

Additional information
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