

# SARS-CoV-2–Specific Immune Responses in Patients With Postviral Syndrome After Suspected COVID-19

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## Abstract

### Background and Objectives

Millions of Americans were exposed to SARS-CoV-2 early in the pandemic but could not get diagnosed with COVID-19 due to testing limitations. Many have developed a postviral syndrome (PVS) including neurologic manifestations similar to those with postacute sequelae of SARS-CoV-2 infection (Neuro-PASC). Owing to those circumstances, proof of SARS-CoV-2 infection was not required for evaluation at Northwestern Medicine's Neuro COVID-19 clinic. We sought to investigate clinical and immunologic findings suggestive of SARS-CoV-2 exposure in patients with PVS.

### Methods

We measured SARS-CoV-2–specific humoral and cell-mediated immune responses against Nucleocapsid and Spike proteins in 29 patients with PVS after suspected COVID-19, 32 confirmed age-matched/sex-matched Neuro-PASC (NP) patients, and 18 unexposed healthy controls. Neurologic symptoms and signs, comorbidities, quality of life, and cognitive testing data collected during clinic visits were studied retrospectively.

### Results

Of 29 patients with PVS, 12 (41%) had detectable humoral or cellular immune responses consistent with prior exposure to SARS-CoV-2. Of 12 PVS responders (PVS<sup>+</sup>), 75% harbored anti-Nucleocapsid and 50% harbored anti-Spike responses. Patients with PVS<sup>+</sup> had similar neurologic symptoms as patients with NP, but clinic evaluation occurred 5.3 months later from the time of symptom onset (10.7 vs 5.4 months;  $p = 0.0006$ ). Patients with PVS<sup>+</sup> and NP had similar subjective impairments in quality of life measures including cognitive function and fatigue. Patients with PVS<sup>+</sup> had similar results in objective cognitive measures of processing speed, attention, and executive function and better results in working memory than patients with NP.

### Discussion

Antibody and T-cell assays showed evidence of prior SARS-CoV-2 exposure in approximately 40% of the PVS group. Three-quarters of patients with PVS<sup>+</sup> had detectable anti-Nucleocapsid and one-half anti-Spike responses, highlighting the importance of multitargeted COVID-19 immunologic evaluation and the limitations of commercially available diagnostic tests. Despite their persistent symptoms, lack of COVID-19 diagnosis likely delayed clinical care in patients with PVS. Our data suggest that millions of Americans presenting with PVS resembling Neuro-PASC were indeed exposed to SARS-CoV-2 at the beginning of the pandemic, and they deserve the same access to care and inclusion in research studies as patients with NP with confirmed COVID-19 diagnosis.

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## Glossary

HC = healthy controls; IQR = interquartile range; PBMC = peripheral blood mononuclear cell; PROMIS = Patient-Reported Outcomes Measurement Information System; PVS = postviral syndrome.

## Introduction

The COVID-19 pandemic has led to over 103 million confirmed cases and more than 1.1 million deaths in the United States as of March 2023.<sup>1</sup> Up to one-third of individuals who survive acute COVID-19 infection will develop symptoms persisting longer than 6 weeks,<sup>2</sup> known as long COVID-19 or postacute sequelae of SARS-CoV-2 infection (PASC). Many individuals with PASC experience neurologic symptoms including cognitive impairment, fatigue, autonomic disturbances, myalgia, headache, and other pain syndromes,<sup>3</sup> termed Neuro-PASC (NP).

Most patients with NP experience mild and transient respiratory symptoms of COVID-19 and do not require hospitalization during the acute infection.<sup>4</sup> However, databases of confirmed infections underestimate the total number of COVID-19 cases due to limited access to testing early in the pandemic<sup>5</sup> or because of testing outside the window of detectable nasopharyngeal viral shedding or seropositivity.<sup>6</sup> This suggests that millions of Americans are experiencing the long-term consequences of COVID-19 without having an official diagnosis of acute disease. Because most post-COVID-19 clinics in the United States are only accepting patients with a prior positive SARS-CoV-2 test result,<sup>7</sup> individuals experiencing postviral syndrome (PVS) identical to PASC are left without specialized care when exposure to SARS-CoV-2 is not confirmed. Furthermore, the same people are also excluded from participation to research studies on PASC. Therefore, we sought to investigate clinical and immunologic measures suggestive of SARS-CoV-2 exposure in patients with PVS seen at Northwestern Medicine's Neuro COVID-19 clinic.

## Methods

### Participants and Study Design

Research objectives were to investigate evidence of SARS-CoV-2-specific adaptive immune responses in a subset of participants with patients with PVS suspected of having COVID-19 despite lacking laboratory evidence of prior SARS-CoV-2 infection by clinically available diagnostic testing, including RT-PCR and serology tests. Subjects were not randomized, and investigators were not blinded to subject groups when performing experiments and analyzing data.

Since May 2020, our Neuro COVID-19 clinic has cared for patients presenting with neurologic manifestations of PASC. Because PASC was a new syndrome not yet defined, we accepted patients complaining of any type of neurologic

manifestations associated with SARS-CoV-2 infection as well as patients with suspected infection, but without positive COVID-19 test. We did not require physician referral, and our only exclusion criteria were the absence of any neurologic symptoms (e.g., patients complaining only of shortness of breath after COVID-19). Patients were evaluated in person or through telemedicine.

Outpatients seen at Northwestern Medicine's Neuro COVID-19 clinic between June 2020 and April 2022 were enrolled in this study, including 23 unvaccinated and 6 vaccinated patients with PVS. PVS cases were age-matched and sex-matched with 32 neurologic postacute sequelae of COVID-19 (Neuro-PASC, NP) with laboratory-confirmed COVID-19 by RT-PCR or serology from the same clinic as positive controls for immune responses. Patients with NP had persistent neurologic symptoms for at least 6 weeks from onset.<sup>3,8</sup> This definition is more stringent than that of the CDC that was formulated after the opening of the Neuro COVID-19 clinic and only requires symptoms lasting more than 4 weeks.<sup>9</sup> Our patients also fit the subsequent WHO criteria of long COVID-19 as well as the PASC criteria from NIH.<sup>10,11</sup> In addition, 18 healthy controls (HCs) consisting of individuals with no known exposure to SARS-CoV-2 or a positive test for COVID-19 were also enrolled.

### Standard Protocol Approvals, Registrations, and Patient Consents

This study was approved by the Institutional Review Board at Northwestern University (STU00212583). All subjects provided informed consent. Samples were deidentified when collected.

### Procedures

#### Plasma and PBMC Collection

In total, 30 mL of venous blood was collected in sodium heparin tubes (BD Biosciences) from participants. Blood samples were processed to isolate plasma and peripheral blood mononuclear cells (PBMCs) and stored as previously described.<sup>12</sup>

#### Evaluation of Quality of Life and Cognition

Patients with PVS and NP completed the Patient-Reported Outcomes Measurement Information System (PROMIS) as the Computer Adaptive Test for the following domains: cognitive function v2.0, fatigue v1.0, sleep disturbance v1.0, anxiety v1.0, and depression v1.0. NIH Toolbox v2.1 was administered to assess the following cognitive domains: processing speed (pattern comparison processing speed test), attention (inhibitory control and attention test), executive function (dimensional change card sort test), and working memory (list sorting working memory test).<sup>13</sup> PROMIS and

NIH Toolbox were completed as part of the patient's clinic visit with results as a T-score based on a normative US reference population with an average/median of 50 and a SD of 10. Patients seen through telemedicine completed PROMIS questionnaires by the time of the clinic visit, and NIH Toolbox was scheduled in person shortly after.

### Nucleocapsid and Spike RBD IgG ELISA

Antigen-specific total antibody titers against SARS-CoV-2 Nucleocapsid (N) protein and Spike receptor-binding domain (S-RBD) were measured using ELISA as previously described.<sup>12</sup> In brief, plasma samples were serially diluted 25-fold to  $4.4 \times 10^6$ -fold on plates coated with 1  $\mu\text{g}/\text{mL}$  of SARS-CoV-2 N or S-RBD protein. N and S-RBD proteins were produced at the Northwestern Recombinant Protein Production Core by Dr. Sergii Pshenychnyi using plasmids under HHSN272201400008C obtained from BEI Resources, NIAID, NIH: Vector pCAGGS containing the SARS-related coronavirus 2, Wuhan-Hu-1 nucleocapsid gene (NR-53507), and Spike RBD (NR-52309). The limit of detection for ELISA anti-N and S-RBD IgG end point titers was defined as double the average signal detected in 5 banked healthy control samples obtained in 2019 before the COVID-19 pandemic.

### SARS-CoV-2 Peptide Antigens

Peptide arrays of SARS-CoV-2 Nucleocapsid (N, NR-52404) and Spike (S, NR-52402) were obtained from BEI Resources, NIAID, NIH. A total of 59 N peptides and 181 S peptides with each peptide being 13-17aa in length were dissolved in either sterile H<sub>2</sub>O or 50% sterile H<sub>2</sub>O-DMSO and combined into respective N and S pools at a stock concentration of 1 mg/mL. Peptide pools were diluted to a final concentration of 2  $\mu\text{g}/\text{mL}$  in all assays.

### Cell Stimulation and IFN- $\gamma$ ELISPOT

IFN- $\gamma$  ELISPOT assays in response to N and S peptide pools were performed and quantified as previously described.<sup>12</sup> The threshold for positive responses by IFN- $\gamma$  ELISPOT was set above the greatest number of spot-forming units per  $10^6$  PBMCs in HC samples after stimulation with SARS-CoV-2 peptide pools, specifically  $>2.1$  standard deviations above the HC mean for N pool and  $>2.9$  standard deviations above the HC mean for S pool.

### Statistical Analysis

Clinical data were collected and managed using Research Electronic Data Capture. Variables were summarized as the number of patients (frequency), mean (SD) for normally distributed variables, and median (interquartile range) for non-normally distributed variables. Two-group comparisons in clinical data were assessed using the Fisher exact test for categorical data, unpaired *t* test for normally distributed continuous variables, and Mann-Whitney *U* test for non-normally distributed continuous variables. The one-sample Wilcoxon signed-rank test was used to determine whether the median T-scores for PROMIS and NIH Toolbox domains differed from the normative US population median T-score of

50. ELISA IgG end point titers were compared across groups through the Kruskal-Wallis test with the uncorrected Dunn test. Differences in IFN- $\gamma$  ELISPOT responses were determined by two-way ANOVA with the Fisher least significant difference test for multiple comparisons. Simple linear regression was performed to assess for a linear relationship between SARS-CoV-2 immune responses and time from symptom onset to sample collection for PVS and NP groups. Associations between immune responses and NIH Toolbox scores were determined using Spearman correlation. Two-sided  $p \leq 0.05$  was considered significant, and all analyses were performed using GraphPad Prism version 9.4.1.

### Data Availability

Anonymized data are available by request to any qualified investigator.

## Results

### Demographics of PVS and NP Groups

The median age of patients with PVS was 42.9 years, 93% were female, 93% were White, none were Hispanic (Table 1), and there was no significant difference compared with the NP group. By definition, none of the patients with PVS ever tested positive for COVID-19 by clinical diagnostic assays, and 22 (76%) and 23 (79%) tested negative by RT-PCR and serology, respectively. By contrast, all 32 patients with NP had a documented positive COVID-19 test with 28 (88%) testing positive by RT-PCR and 20 (63%) testing positive by serology. Six (21%) of the PVS cases were vaccinated against SARS-CoV-2 before sample collection.

### SARS-CoV-2-Specific Immune Responses Are Observed in a Subset of PVS Cases

Virus-specific immune responses indicative of prior exposure to SARS-CoV-2 were measured by Nucleocapsid or Spike ELISA and IFN- $\gamma$  ELISPOT. Anti-Nucleocapsid antibodies were detected in 5 of 29 PVS cases (Figure 1A), while Spike Receptor-Binding Domain (RBD) antibodies were detected in two of the 23 unvaccinated patients with PVS (Figure 1B). Although median end point titers between PVS and unexposed healthy control (HC) groups were similar, we identified several patients who exhibited antibody responses against SARS-CoV-2 Nucleocapsid or Spike proteins. Confirmed Neuro-PASC (NP) had greater anti-Nucleocapsid and anti-Spike RBD IgG end point titers compared with PVS and HC groups. Anti-Nucleocapsid IgG titers did not show a linear relationship with time from COVID-19 onset to sample collection for PVS and NP (eFigure 1, [links.lww.com/NXI/A886](https://links.lww.com/NXI/A886)). Only one patient with PVS was seropositive for both anti-Nucleocapsid and anti-Spike RBD antibodies (eTable 1, [links.lww.com/NXI/A887](https://links.lww.com/NXI/A887)).

We then used IFN- $\gamma$  ELISPOT to measure T-cell responses against SARS-CoV-2 structural proteins on a per-cell basis. Positive IFN- $\gamma$  response to the Nucleocapsid peptide pool by

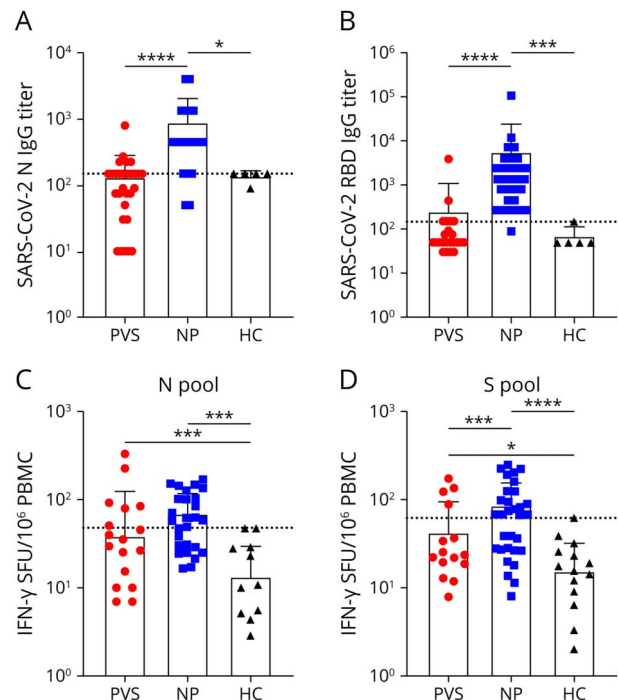
**Table 1** Study Subjects' Demographics in Patients With Postviral Syndrome (PVS) and Neuro-PASC (NP)

	All PVS	NP	<i>p</i> , all PVS vs NP
<b>n</b>	29	32	
<b>Age, y (median (IQR))</b>	42.9 (37.5–54.6)	46.4 (39.9–53.7)	0.54
<b>Sex, n (%)</b>			0.15
<b>Male</b>	2 (7)	7 (22)	
<b>Female</b>	27 (93)	25 (78)	
<b>Race, n (%)</b>			0.41
<b>White</b>	27 (93)	27 (84)	
<b>Black or African American</b>	1 (3)	1 (3)	
<b>Asian</b>	0 (0)	3 (9)	
<b>Other</b>	1 (3)	1 (3)	
<b>Ethnicity, n (%)</b>			0.11
<b>Not Hispanic or Latino</b>	29 (100)	28 (88)	
<b>Hispanic or Latino</b>	0 (0)	4 (12)	
<b>Visit type, n (%)</b>			1
<b>In-person</b>	20 (69)	22 (69)	
<b>Televisit</b>	9 (31)	10 (31)	
<b>SARS-CoV-2 RT-PCR, n (%)</b>			<b>&lt;0.0001</b>
<b>Positive</b>	0 (0)	28 (88)	
<b>Negative</b>	22 (76)	3 (9)	
<b>Not performed</b>	7 (24)	1 (3)	
<b>SARS-CoV-2 serology, n (%)</b>			<b>&lt;0.0001</b>
<b>Positive</b>	0 (0)	20 (63)	
<b>Negative</b>	23 (79)	2 (6)	
<b>Not performed</b>	6 (21)	10 (31)	
<b>Either RT-PCR or serology positive, n (%)</b>	0 (0)	32 (100)	<b>&lt;0.0001</b>

Bold indicates statistically significant *p* values.

ELISPOT relative to HC subjects was detected in 6 of 24 PVS cases tested (Figure 1C). Patients with PVS and NP had similar levels of Nucleocapsid-specific T-cell activation, while both groups showed enhanced IFN- $\gamma$  production relative to HC. In addition, 4/18 patients with PVS mounted Spike-specific IFN- $\gamma$  responses (Figure 1D). Two PVS cases were both Nucleocapsid and Spike positive by IFN- $\gamma$  ELISPOT. In total, SARS-CoV-2-specific immune responses were present in 12/29 (41%) of the PVS cases (eTable 1, [links.lww.com/NXI/A887](https://links.lww.com/NXI/A887)), hereafter termed PVS responders (PVS<sup>+</sup>). Of the 12 patients with PVS<sup>+</sup>, 9 (75%) tested positive for Nucleocapsid-specific antibody or T-cell

**Figure 1** SARS-CoV-2-Specific Antibody and T-Cell Responses in Patients With Postviral Syndrome (PVS)



(A) Plasma anti-Nucleocapsid IgG ELISA and (B) anti-Spike Receptor-Binding Domain (RBD) IgG ELISA. (C) IFN- $\gamma$  ELISPOT response after stimulation with SARS-CoV-2 Nucleocapsid (N) and (D) Spike peptide pools. Only unvaccinated subjects were examined for anti-Spike responses in B and D. Horizontal dotted line in A, B = limit of detection. Horizontal dotted line in C, D = threshold for positive IFN- $\gamma$  ELISPOT response. \**p* < 0.05, \*\*\**p* < 0.001, \*\*\*\**p* < 0.0001 by Kruskal-Wallis test for A, B and two-way ANOVA for C, D.

responses and 6/12 (50%) tested positive for anti-Spike antibody or T-cell responses.

### PVS<sup>+</sup> Are Clinically Similar to Patients With NP but Present to Clinic Later From Symptom Onset

PVS responders were evaluated in our clinic at a median of 10.7 months, which was significantly longer than the median time from symptom onset to clinic visit of 5.4 months in patients with NP. Based on this difference between patients with PVS and NP, we assessed whether the time elapsed since symptom onset correlated with adaptive immunity to Nucleocapsid (eFigure 1, [links.lww.com/NXI/A886](https://links.lww.com/NXI/A886)) or Spike (data not shown), both of which were not significant. Clinical findings were further compared between PVS and NP groups (Table 2). The total average subjective impression of recovery compared with pre-COVID-19 baseline was 65.6% in patients with PVS<sup>+</sup>, similar to 62.2% for the NP group. The average number of symptoms attributed to COVID-19 in patients with PVS<sup>+</sup> was 6.0, and 92% reported at least 4 neurologic symptoms which was not different from the NP group. The only difference in symptoms observed between patients with PVS<sup>+</sup> and PVS<sup>-</sup> was numbness and tingling (25% in PVS<sup>+</sup> vs 71% in PVS<sup>-</sup>; *p* = 0.03). The frequency of abnormal neurologic examination in PVS<sup>+</sup> did not differ compared with PVS<sup>-</sup> or NP groups.

**Table 2** Neurologic Symptoms and Signs Attributed to Postviral Syndrome or Neuro-PASC

	All PVS	PVS responders (PVS <sup>+</sup> )	PVS nonresponders (PVS <sup>-</sup> )	<i>p</i> , PVS <sup>+</sup> vs PVS <sup>-</sup>	NP	<i>p</i> , PVS <sup>+</sup> vs NP
Time from symptom onset to clinic visit (mo, median (IQR))	7.7 (6.3–12.6)	10.7 (6.6–15.3)	7.5 (6.1–12.6)	0.39	5.4 (2.6–7.7)	<b>0.0006</b>
Subjective impression of recovery compared with pre-COVID-19 baseline (mean % (1 SD))	57.2 (26.1)	65.6 (26.1) n = 11	51.0 (25.2) n = 16	0.16	62.2 (17.8) n = 30	0.63
Number of neurologic manifestations/symptoms attributed to COVID-19 (mean [SD])	6.0 (2.7)	6.0 (2.3)	5.9 (3.0)	0.95	6.2 (2.5)	0.85
<b>Neurologic symptom n (%)</b>						
≥4	25 (86)	11 (92)	14 (82)	0.62	28 (88)	1
Brain fog	26 (90)	10 (83)	16 (94)	0.55	28 (88)	0.66
Headache	22 (76)	8 (67)	14 (82)	0.40	27 (84)	0.23
Myalgia	18 (62)	9 (75)	9 (53)	0.27	19 (59)	0.49
Anosmia or dysgeusia	18 (62)	9 (75)	9 (53)	0.27	26 (81)	0.69
Numbness/tingling	15 (52)	3 (25)	12 (71)	<b>0.03</b>	14 (44)	0.32
Dizziness	14 (48)	6 (50)	8 (47)	1	17 (53)	1
Tinnitus	12 (41)	5 (42)	7 (41)	1	10 (31)	0.72
Pain other than chest	11 (38)	7 (58)	4 (24)	0.12	13 (41)	0.33
Blurred vision	10 (34)	5 (42)	5 (29)	0.69	9 (28)	0.48
Seizure	2 (7)	1 (8)	1 (6)	1	0 (0)	0.27
Movement disorder <sup>a</sup>	2 (7)	1 (8)	1 (6)	1	0 (0)	0.27
Focal motor deficit <sup>b</sup>	1 (3)	1 (8)	0 (0)	0.41	0 (0)	0.27
Ataxia	1 (3)	1 (8)	0 (0)	0.41	0 (0)	0.27
<b>Other symptom n (%)</b>						
Fatigue	28 (97)	11 (92)	17 (100)	0.41	27 (84)	1
Shortness of breath	19 (66)	8 (67)	11 (65)	1	15 (47)	0.32
Depression/Anxiety	16 (55)	7 (58)	9 (53)	1	20 (63)	1
Chest pain	16 (55)	6 (50)	10 (59)	0.72	9 (28)	0.28
Dysautonomia <sup>c</sup>	11 (38)	3 (25)	8 (47)	0.27	11 (34)	0.72
Insomnia	10 (34)	5 (42)	5 (29)	0.69	20 (63)	0.31
GI symptoms <sup>d</sup>	9 (31)	5 (42)	4 (24)	0.42	10 (31)	0.72
<b>Sign n (%)</b>						
Abnormal exam	12 (41)	5 (42)	7 (41)	1	14/30 (47)	1
Short-term memory deficit	8 (28)	4 (33)	4 (24)	0.68	6/30 (20)	0.43
Sensory dysfunction <sup>e</sup>	6/20 (30)	2/7 (29)	4/13 (31)	1	5/22 (23)	1
Attention deficit	4 (14)	0 (0)	4 (24)	0.12	4/30 (13)	0.31
Gait dysfunction	3 (10)	1 (8)	2 (12)	1	0/30 (0)	0.29
Cerebellar dysfunction	2 (7)	1 (8)	2 (12)	1	0/30 (0)	0.29
Movement disorder	1 (3)	1 (8)	0 (0)	0.41	0/30 (0)	0.29
Motor dysfunction	0 (0)	0 (0)	0 (0)	1	1/30 (3)	1

Bold indicates statistically significant *p* values.

<sup>a</sup> PVS<sup>+</sup>: Positional tremor in bilateral upper extremities (1). PVS<sup>-</sup>: Facial twitches (1). NP: none.

<sup>b</sup> PVS<sup>+</sup>: Poor coordination and weakness with left hand (1). PVS<sup>-</sup>: None. NP: none.

<sup>c</sup> PVS<sup>+</sup>: Self-reported variation of heart rate (2), other nondefined attributed to variation of heart rate and blood pressure (1). PVS<sup>-</sup>: POTS (4), syncope (2), orthostatic hypotension (1), self-reported variation of heart rate (1). NP: Self-reported variation of heart rate (5), self-reported variation of blood pressure (2), orthostatic hypotension (1), POTS (1), other nondefined attributed to variation of heart rate and blood pressure (3).

<sup>d</sup> PVS<sup>+</sup>: Vomiting (1), diarrhea (5). PVS<sup>-</sup>: Nausea (3), vomiting (1), diarrhea (1). NP: Nausea (6), vomiting (1), diarrhea (5).

<sup>e</sup> Evaluated for in person visits only.

In addition, the frequency of abnormal neurologic examination did not differ significantly between in-person and telehealth visits (data not shown).

Comparison of comorbidities between groups showed that the frequency of conditions existing before suspected or confirmed COVID-19 was not significantly different between PVS<sup>+</sup> and NP groups (eTable 2, [links.lww.com/NXI/A888](https://links.lww.com/NXI/A888)). Most patients had preexisting comorbidities before suspected or confirmed COVID-19 (58% in PVS<sup>+</sup> and 72% in NP). Most commonly, comorbidities were depression/anxiety (42% in PVS<sup>+</sup> and 28% in NP), headache (25% in PVS<sup>+</sup> and 19% in NP), hypertension (25% in PVS<sup>+</sup> and 16% in NP), neuropsychiatric disease (25% in PVS<sup>+</sup> and 25% in NP), dyslipidemia (25% in PVS<sup>+</sup> and 22% in NP), and autoimmune disease (10% in PVS<sup>+</sup> and 19% in NP). Three cases of Lyme disease and one case of prior Chikungunya viral infection were noted in PVS<sup>-</sup> but were not seen in patients with PVS<sup>+</sup> or NP. Overall, preexisting comorbidities in patients with PVS<sup>+</sup> resembled that of the NP group.

### Quality of Life Measures and Cognitive Testing in PVS Responders and Nonresponders

Patients completed PROMIS quality of life measures at the time of the clinic visit to quantify subjective impression of their cognitive function, fatigue, sleep disturbance, anxiety, and depression. No differences in PROMIS T-scores were observed between patients with PVS<sup>+</sup> and PVS<sup>-</sup> (Figure 2A). Cognitive function and fatigue T-scores were significantly worse than the normative population for PVS<sup>+</sup> and PVS<sup>-</sup> groups, while anxiety was only significantly different from the normative population in PVS<sup>+</sup> (Figure 2C). Similar to patients with PVS<sup>+</sup>, subjective impression of cognitive function, fatigue, anxiety, and depression T-scores were significantly worse from the normative population for NNP subjects. Moreover, quality of life measures were similarly impaired in patients with NP and did not differ from the PVS<sup>+</sup> group.

NIH Toolbox was administered to objectively assess for cognitive dysfunction in the following domains: processing speed, attention, executive function, and working memory. PVS<sup>+</sup> and PVS<sup>-</sup> groups did not show any differences in NIH Toolbox T-scores (Figure 2B). The only difference from the normative population was significantly lower attention T-scores in patients with PVS<sup>+</sup> (Figure 2C,  $p = 0.01$ ), which has been reported previously in a larger group of patients with confirmed NP.<sup>8</sup> Only working memory T-scores were higher in PVS<sup>+</sup> compared with NP ( $p = 0.02$ ). Finally, attention and working memory T-scores were significantly worse than the normative population for the NNP group. Altogether, NIH Toolbox results showed that attention was the most impaired cognitive domain tested for PVS<sup>+</sup> and NP groups.

Overall, our study showed that SARS-CoV-2–specific adaptive immune responses could be detected in a sizeable portion of a PVS group after suspected COVID-19. N-specific IFN- $\gamma$  ELISPOT yielded a positive response in a quarter of PVS

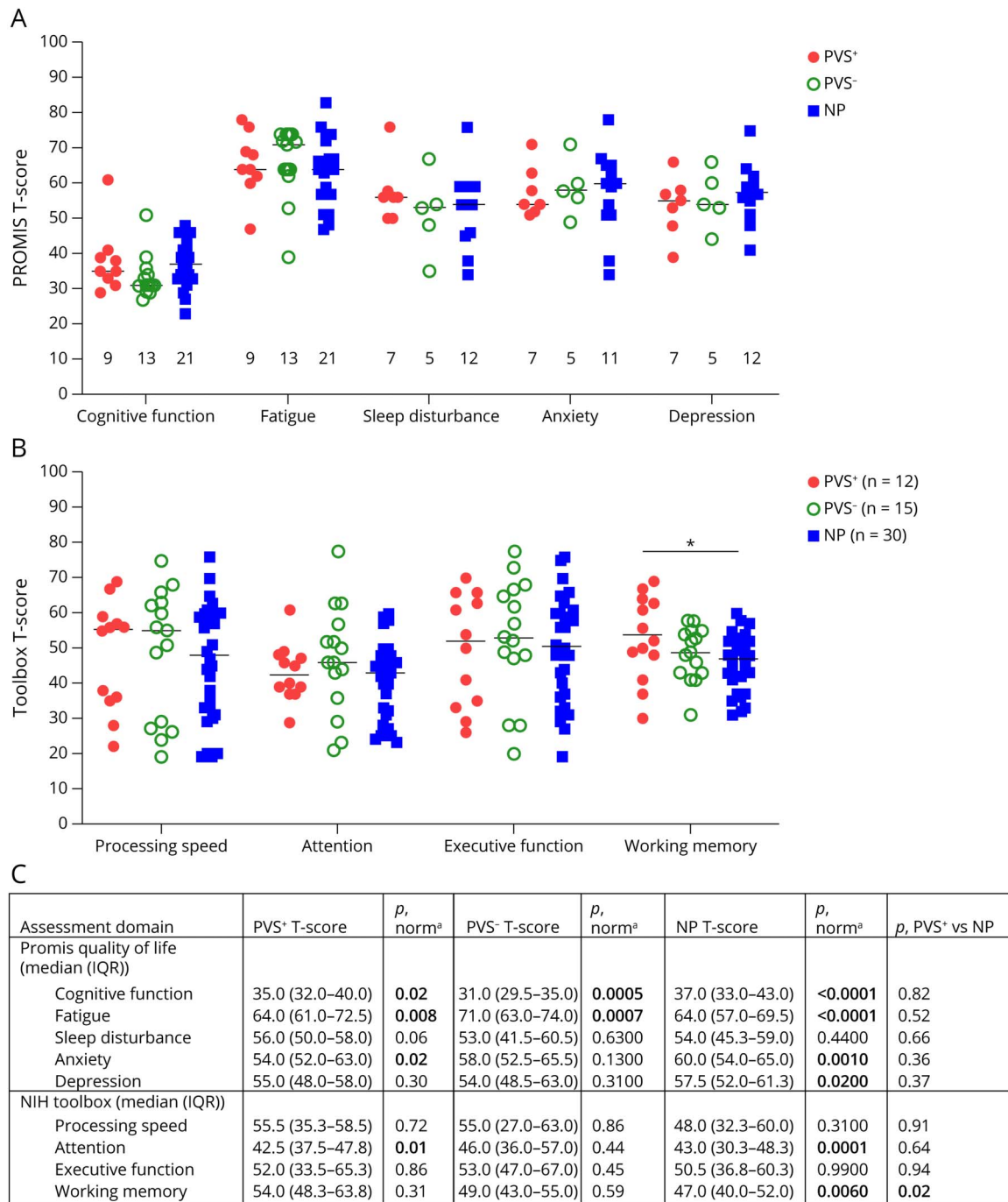
cases tested. Symptoms and preexisting comorbidities did not differ between PVS<sup>+</sup> and NP groups except for dysgeusia being more frequent in patients with NP. Quality of life measures were not different between these 2 groups, and cognitive performance was for the most part similar in PVS<sup>+</sup> compared with NP groups. Collectively, the immune responses and clinical presentation of PVS<sup>+</sup> cases closely resemble patients with COVID-19–confirmed NP.

## Discussion

PASC has become a well-recognized condition often involving the nervous system. However, patients with PVS with clinical manifestations identical to PASC but without confirmed SARS-CoV-2 diagnosis are often unable to access clinical care and are blocked from participating in research studies when a positive COVID-19 test is required. We previously reported that only 19/64 (30%) post-COVID-19 clinics in the United States surveyed would accept to see SARS-CoV-2 laboratory-negative “long haulers.”<sup>7</sup> These barriers to seeking clinical care likely contributed to the five-month delay in the median time from symptom onset to clinic visit in patients with PVS compared with patients with NP. Our findings may have important consequences for a large number of people in the United States. Indeed, it is possible that over 10 million Americans have developed PASC without confirmed COVID-19 diagnosis due to limited testing availability during the first year of the pandemic.<sup>7</sup> Our data show that 41% of patients with PVS consulting at our Neuro COVID-19 clinic have detectable SARS-CoV-2–specific adaptive immune responses, suggesting at least 4 million individuals with PVS resembling PASC in the United States may indeed have detectable immune responses to support a COVID-19 diagnosis. This emphasizes the importance of refining COVID-19 diagnostic testing to increase sensitivity and providing clinical care to individuals with PVS after suspected COVID-19.

We found that virus-specific immune responses were not always present against both SARS-CoV-2 N and S proteins and encompassing both antibody and T-cell assays. It has been previously reported that laboratory-confirmed COVID-19 convalescents can be seronegative for anti-Spike IgG while still having positive T-cell responses against Spike peptides.<sup>14</sup> Others have reported that anti-N antibodies may persist for a shorter amount of time than anti-S antibodies after SARS-CoV-2 infection.<sup>15</sup> However, these studies examine COVID-19 convalescents without distinguishing patients with Neuro-PASC from those without persistent symptoms. In fact, our group has shown that individuals with confirmed Neuro-PASC can have detectable anti-N IgG antibodies and N-specific IFN- $\gamma$  T-cell response over a year postonset,<sup>12</sup> suggesting SARS-CoV-2 Nucleocapsid-specific immune responses may persist in patients with NP for a longer period than in COVID-19 convalescents without lingering symptoms. Similarly, patients with PVS showed an enhanced N-specific IFN- $\gamma$  ELISPOT response

**Figure 2** Quality of Life and Cognitive Measures in Patients With PVS and Neuro-PASC



<sup>a</sup>norm: against normative population median T-score of 50

(A) Normalized T-score values for Patient-Reported Outcomes Measurement Information System (PROMIS) in PVS+, PVS nonresponders (PVS-), and NP subjects with n labeled under corresponding group and domain. (B) Normalized and demographic-matched T-score values for NIH Toolbox cognitive assessment. (C) Tabulated summary of PROMIS and Toolbox scores reported as median and interquartile range with comparisons with the normative population and between PVS+ and NP groups. Scores lower than 50 for PROMIS cognitive function and NIH Toolbox processing speed, attention, executive function, and working memory indicate poor outcomes. Scores higher than 50 for PROMIS fatigue, sleep disturbance, anxiety, and depression indicate poor outcomes. \* $p < 0.05$  by Mann-Whitney for groupwise comparisons and one-sample Wilcoxon signed-rank test comparing with the normative population.

compared with unexposed healthy controls that was not significantly different from patients with NP. The heterogeneity in antiviral humoral vs cellular immune responses supports the inclusion of assays that measure SARS-CoV-2 T cell responses to determine past exposure.

Considering most of the US population has now received Spike COVID-19 vaccines, anti-Spike serologic assays can only be used to support prior SARS-CoV-2 exposure in a limited number of unvaccinated individuals. Moreover, both patients with PVS in this study testing positive for anti-Spike

IgG had previously tested negative by clinical diagnostic testing, suggesting anti-Spike serology assays should not be used alone to diagnose PASC, even in unvaccinated individuals. Therefore, testing for anti-Nucleocapsid antibody responses is crucial for determining past exposure.

Interestingly, 4 of the 5 Nucleocapsid seropositive subjects by our assay previously tested negative for anti-Nucleocapsid IgG by the Abbott serologic test, a qualitative chemiluminescent microparticle immunoassay testing serum or plasma at a single concentration.<sup>16</sup> By contrast, our IgG ELISA is a semiquantitative assay using 12 serial dilutions to determine end point titers, which has a greater dynamic range than commercially available COVID-19 serology testing. We observed a positive IFN- $\gamma$  response against Nucleocapsid protein in 6/24 (25%) patients with PVS tested, and this response was detected in 6/12 (50%) patients with PVS<sup>+</sup>. Collectively, our data suggest that measuring N-specific T-cell and antibody responses may be the most promising assay in supporting a diagnosis of SARS-CoV-2 infection in PVS cases.

When comparing PVS cases with age-matched and sex-matched confirmed NP subjects, we found that the frequency of neurologic signs and symptoms attributed to COVID-19 was for the most part similar between PVS<sup>+</sup> and NP subjects. The WHO COVID-19 case definitions published in December 2020 (used at the time of enrolling the last subject in this study) considers patients with PVS as “probable” cases of SARS-CoV-2 infection when acute symptoms included recent onset of anosmia or dysgeusia.<sup>17</sup> Of the 12 patients with PVS<sup>+</sup>, 9 (75%) met the criteria for probable SARS-CoV-2 infection, while the remaining 3/12 (25%) are suspected cases. Patients with PVS<sup>-</sup> would be classified as 9/17 (53%) probable and 8/17 (47%) suspected SARS-CoV-2 infection. Although we defined persistent symptoms as greater than 6 weeks, the PVS<sup>+</sup> group came to clinic at a median of 10.7 months after onset with 92% reporting at least 4 neurologic symptoms. We have previously shown that patients’ subjective impression of their recovery from NP does not correlate with time from onset.<sup>8</sup> These findings suggest a holistic approach combining clinical and multitargeted immunologic measures is most appropriate for defining a diagnosis of PASC in patients with PVS without a positive RT-PCR or antigen test for SARS-CoV-2 during the acute infection.

Between PVS<sup>+</sup> and PVS<sup>-</sup> groups, the only difference in symptoms was numbness and tingling being more common in the PVS<sup>-</sup> group. Interestingly, PVS<sup>-</sup> subjects were the only cases with a medical history of Lyme disease, Chikungunya infection, and traumatic brain injury predating the onset of suspected COVID-19. While this only encompasses one-third of this group, these preexisting conditions may contribute to persistent neurologic symptoms and could potentially have been exacerbated by another viral infection. Our data show no evidence that these individuals were exposed to SARS-CoV-2.

We did not observe any differences in PROMIS and NIH Toolbox measures between PVS<sup>+</sup> and PVS<sup>-</sup> subjects by groupwise comparison. However, when comparing each group with the normative US population, significant differences were seen for quality of life measures in domains of cognitive function and fatigue in both groups, while anxiety and cognitive measure of attention were only significantly worse for PVS<sup>+</sup> subjects. This impairment in attention on the NIH Toolbox relative to the normative population has been previously described in a larger group of 315 Neuro-PASC patients,<sup>8</sup> suggesting that PVS<sup>+</sup> subjects exhibit attentional deficits as seen in confirmed patients with NP. Groupwise comparisons revealed that working memory T-scores were higher in PVS<sup>+</sup> cases compared with NP, although both groups scored on average within 0.5 standard deviations of the normative population mean. Prior research has shown that working memory task on the NIH Toolbox is not impaired in nonhospitalized patients with NP,<sup>8</sup> consistent with our findings in PVS<sup>+</sup> and NP subjects.

The COVID-19 pandemic has highlighted important disparities in access to health care in the United States affecting underserved populations suffering from both acute and chronic manifestations of SARS-CoV-2 infection.<sup>18,19</sup> The group of patients with PVS included in this study was 93% White and 100% non-Hispanic. However, this population constituted predominantly of female patients in their forties who do not have a confirmed diagnosis of COVID-19 has also been underserved. Indeed, those patients, who at one point comprised half of our clinic population,<sup>3</sup> have experienced much rejection and stigma by the medical establishment and are underrepresented in the medical literature.<sup>20,21</sup> Unfortunately, suppressing scientific publications including patients with PVS only extends their dismissal.

This study is limited to a small sample size of PVS cases. Many of these patients suspected having COVID-19 in early 2020 when obtaining accurate diagnostic testing within the appropriate timeframe of symptom onset was challenging. Subjects were also enrolled at varying times from symptom onset based on when they presented to the clinic. It is possible that some of our PVS<sup>-</sup> cases might have tested positive for SARS-CoV-2-specific immune responses if blood samples were collected closer to symptom onset. Future studies should consider screening patients with a suspected history of COVID-19 for viral-specific immune responses by multiple methods and antigen targets.

This study measures humoral and cellular SARS-CoV-2-specific immune responses in patients with PVS with suspected COVID-19 to identify a subset with evidence of prior exposure after having no history of a positive test. We show that a single measure of virus-specific adaptive immunity is not sufficient for identifying all PVS responders and that a comprehensive approach including clinical evaluation and measurement of the N-specific antibody and T-cell responses may offer the best possibility of determining prior SARS-CoV-2 exposure. Future studies will be necessary to elucidate the heterogeneity and



kinetics of SARS-CoV-2–specific immune responses in patients with NP and refine diagnostic testing for suspected COVID-19 cases lacking positive RT-PCR or antigen testing during the acute infection, particularly for individuals with persistent symptoms resembling PASC. Patients with PVS often present with similar clinical manifestations as confirmed patients with NP, suggesting that a positive result by commercially available SARS-CoV-2 diagnostic test should not be a prerequisite for accessing care. Patients with PVS may benefit from the same clinical care as confirmed patients with NP, and the absence of a positive SARS-CoV-2 test should not preclude or delay treatment.

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## Appendix (continued)

Name	Location	Contribution
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