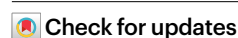


# SARS-CoV-2 reservoir in post-acute sequelae of COVID-19 (PASC)

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Millions of people are suffering from Long COVID or post-acute sequelae of COVID-19 (PASC). Several biological factors have emerged as potential drivers of PASC pathology. Some individuals with PASC may not fully clear the coronavirus SARS-CoV-2 after acute infection. Instead, replicating virus and/or viral RNA—potentially capable of being translated to produce viral proteins—persist in tissue as a ‘reservoir’. This reservoir could modulate host immune responses or release viral proteins into the circulation. Here we review studies that have identified SARS-CoV-2 RNA/protein or immune responses indicative of a SARS-CoV-2 reservoir in PASC samples. Mechanisms by which a SARS-CoV-2 reservoir may contribute to PASC pathology, including coagulation, microbiome and neuroimmune abnormalities, are delineated. We identify research priorities to guide the further study of a SARS-CoV-2 reservoir in PASC, with the goal that clinical trials of antivirals or other therapeutics with potential to clear a SARS-CoV-2 reservoir are accelerated.

A subset of individuals infected with the coronavirus SARS-CoV-2 develop new symptoms or sequelae that do not resolve for months or years. This condition is known as Long COVID or post-acute sequelae of COVID-19 (PASC)<sup>1</sup>. Based on the Census Bureau Household Pulse Survey, the US Centers for Disease Control and Prevention estimates that ~6% of US adults suffer from new symptoms lasting three or more months after contracting COVID-19 (ref. 2). Of those, 80.7% state that their new symptoms limit their ability to carry out day-to-day activities; 26.2% say that their activity is limited ‘a lot’. Estimates place the total US economic cost of PASC at approximately \$743 billion per year, including reduced quality of life, lost earnings and increased medical spending<sup>3</sup>.

Common PASC symptoms include fatigue, flu-like symptoms, autonomic dysfunction, trouble with memory or concentration and post-exertional malaise<sup>4</sup>. However, more than 200 PASC symptoms have been documented and symptom presentation can differ from person to person<sup>5,6</sup>. In addition, many individuals with PASC report symptoms of fluctuating severity or a relapsing/remitting nature<sup>7</sup>. PASC can occur in children, with an incidence of up to 25% of cases in earlier COVID-19 waves<sup>8</sup>, and more recent reports suggesting that roughly 6% of children infected with SARS-CoV-2 meet PASC criteria<sup>9</sup>. The most severe post-COVID-19 sequelae in children is multisystem inflammatory syndrome (MIS-C): a sometimes fatal SARS-CoV-2-related inflammatory disorder that has been defined as part of the PASC spectrum. More than

9,300 children have developed MIS-C in the USA alone<sup>10</sup>. Overall, the tremendous disability and economic burden of PASC on both adult and pediatric populations requires that core biological drivers of the disease process be rapidly delineated.

Several biological trends are emerging as primary potential drivers of PASC pathology. One is that some individuals with PASC may not fully clear SARS-CoV-2 after initial infection. Instead, replicating virus and/or viral RNA—potentially capable of being translated to produce viral proteins—may persist in patients' tissues in a 'reservoir'. SARS-CoV-2 is a positive-sense single-stranded RNA virus from the Coronaviridae family. There is precedence for the persistence of other single-stranded RNA viruses after acute illness. RNA from Ebola virus<sup>11–13</sup>, Zika virus<sup>14</sup>, enteroviruses<sup>15,16</sup> and measles virus<sup>17,18</sup> has been identified in tissue obtained months or years after initial infection. In multiple instances, these viral reservoirs have been shown to be capable of driving chronic disease<sup>19,20</sup>. In the case of Ebola virus disease, new outbreaks have been sparked by individuals carrying persistent Ebola virus years after acute illness<sup>21,22</sup>, and there are multiple reports of sexual transmission of Zika virus many months after recovery from acute disease<sup>23</sup>.

In this Review, we explore evidence for SARS-CoV-2 reservoir in PASC and provide context on interpretation of the findings. We delineate mechanisms by which a SARS-CoV-2 reservoir may contribute to PASC pathology and identify central research priorities and methods to guide the continued study of SARS-CoV-2 persistence in PASC. If used synergistically, these approaches should reveal biomarkers and therapeutic candidates for PASC clinical trials including immunomodulators and direct-acting and host-directed antivirals.

## SARS-CoV-2 is capable of persistence in many body sites

Autopsy and tissue biopsy studies have identified SARS-CoV-2 RNA and protein in a wide range of tissue types collected weeks or months after acute COVID-19 (refs. 24–30). Most of these studies were not designed to measure PASC symptoms, but nevertheless provide evidence that SARS-CoV-2 is capable of persistence in numerous reservoir sites (Table 1). One autopsy study identified SARS-CoV-2 RNA and protein in dozens of body tissues and brain obtained at least 31 d and up to 230 d after COVID-19 symptom onset<sup>31</sup>. Over 50% of these cases had persistent RNA in lymph nodes from the head and neck, and from the thorax, sciatic nerve, ocular tissue and in most sampled regions of the central nervous system (CNS) including the cervical spinal cord, brainstem and olfactory nerve. In one individual who died 230 d after mild COVID-19, SARS-CoV-2 RNA was identified in multiple anatomical sites, including several brain regions. Subgenomic RNA—a potential marker of recent viral replication—was identified in tissues after acute COVID-19, including in multiple tissues of a case at day 99—indicating that viral replication may occur in non-respiratory tissues for several months. Another study identified SARS-CoV-2 RNA in 80% of lung tissue samples obtained from individuals up to 174 d after COVID-19 onset<sup>32</sup>.

SARS-CoV-2 RNA or protein has been identified in tissue months after initial illness despite negative results via standard nasopharyngeal PCR testing and/or a lack of detection in peripheral blood from the same individual<sup>31,33</sup>. These observations suggest that SARS-CoV-2 persistence occurs largely in tissues. Indeed, most human tissue types are dense with cells expressing the angiotensin 2 (ACE2) and transmembrane serine protease 2 (TMPRSS2) receptors SARS-CoV-2 uses for cell entry. A similar pattern has been documented with other RNA viruses associated with chronic sequelae in a subset of survivors<sup>34–36</sup>. Immune responses against SARS-CoV-2 RNA and protein, including those indicative of persistence, can also be localized to tissue and are not necessarily apparent in the blood of the same individual<sup>37</sup>.

## SARS-CoV-2 reservoir in PASC

A major gap in the field is the absence of PASC-specific autopsy data. Thus, most evidence for SARS-CoV-2 reservoir in individuals with PASC

comes from: (1) tissue biopsy studies; (2) studies of SARS-CoV-2 proteins in plasma; and (3) studies using features of the adaptive immune response to infer the presence of a SARS-CoV-2 reservoir in tissues. For example, to investigate the intestinal mucosa as a SARS-CoV-2 reservoir site in PASC, Zollner et al. performed a tissue biopsy study of individuals with inflammatory bowel disease undergoing endoscopy<sup>38</sup>. Despite mild acute infections, 70% of participants harbored SARS-CoV-2 RNA in intestinal mucosal tissue and 52% had nucleocapsid (N) protein in intestinal epithelium ~7 months following COVID-19. Viral RNA and protein persistence were unrelated to the severity of acute COVID-19 or immunosuppressive therapy but were associated with PASC symptoms. Another team detected RNA encoding SARS-CoV-2 spike protein in colorectal tissue collected from five patients with PASC from 158 days to 676 days following the initial COVID-19 illness. These patients were part of a larger group that exhibited evidence of increased T cell activation in the gut and spinal cord via whole-body PET imaging, compared with patients who had recovered from COVID-19 and pre-pandemic control participants<sup>24</sup>. Goh et al. identified SARS-CoV-2 RNA and N protein in the skin, appendix and breast tissue of two individuals who exhibited PASC symptoms 163 and 426 d after acute COVID-19 disease<sup>39</sup>. SARS-CoV-2 RNA and protein were also detected in olfactory mucosa samples 110–196 d after symptom onset in three patients with negative results for nasopharyngeal swab PCR with reverse transcription (RT–PCR) but ongoing anosmia<sup>27</sup>.

Multiple studies have identified SARS-CoV-2 proteins in PASC plasma months or even >1 year after acute COVID-19. This protein is likely derived from PASC tissue reservoir sites, but 'leaks' into the circulation where it can be measured. In a study restricted to unvaccinated individuals, Schultheiß et al. detected SARS-CoV-2 S1 protein in the plasma of approximately 64% of PASC study participants recruited at a median of 8 months (range 1–17 months) after acute COVID-19, but only in approximately 35% of convalescent control patients<sup>40</sup>. Using an optimized ultrasensitive single-molecule array (Simoa) method, Swank et al. identified spike, S1 or N protein in ~65% of plasma samples collected from patients with PASC several months after SARS-CoV-2 infection<sup>41</sup>. Spike was detected most often—in 60% of PASC participants up to 12 months after COVID-19 onset, with no spike detected in COVID-19 convalescent control patients. Viral protein was detected at more than one time point in all 12 of the 37 PASC participants from whom the team had obtained longitudinal samples. Additional Simoa analyses in another post-acute cohort<sup>7</sup> including PASC and fully recovered individuals, found that 24% of all post-acute participants had ≥1 detectable SARS-CoV-2 protein in plasma during at least one time point up to 16 months after COVID-19 (ref. 42), with most of these data obtained before participants had received any SARS-CoV-2 vaccine, a potential confounder in such analyses<sup>43</sup>. The presence of persistent protein was associated with more severe initial infection, with the highest prevalence of protein persistence observed in participants who were consistently the most symptomatic (35% of participants with nine symptoms or more). Notably, a subset of convalescent control patients who reported full recovery (18%) also had detectable viral protein in plasma.

In addition to persisting as soluble proteins in circulation, SARS-CoV-2 proteins, including spike, have been detected in PASC plasma in extracellular vesicles (EVs). One team found higher SARS-CoV-2 S1 and N protein in enriched neuron-derived and astrocyte-derived EVs in plasma from patients with PASC than in that from convalescent control participants<sup>44</sup>. Craddock et al. identified spike protein in the plasma of 64% of PASC participants and 29% of convalescent control participants<sup>45</sup>. They additionally found higher total and relative quantity of EV-associated spike protein in the PASC group, and implicated surface heparin sulfate proteoglycan in spike binding. SARS-CoV-2 RNA was identified in 59% of PASC samples and 28% of convalescent control samples, yet only PASC study participants harbored both spike protein and viral RNA

**Table 1 | Identification of SARS-CoV-2 RNA and protein after COVID-19**

	RNA	Protein	PASC symptoms	Location
<b>Tissue (biopsy)</b>				
Goh et al. <sup>39</sup>	✓	S, N	✓	Appendix, skin and breast tissues 163 and 426 d after COVID-19
Zollner et al. <sup>38</sup>	✓	N	✓	Gut mucosa/epithelium tissue ~7 months after COVID-19
deMelo et al. <sup>27</sup>	✓	N	✓	Olfactory neuroepithelium tissue 110–196 d after COVID-19
Gaebler et al. <sup>33</sup>	✓	N	No	Intestinal tissue ~4 months after COVID-19
Cheung et al. <sup>114</sup>	✓	S, N	NM	Colon, appendix, ileum, hemorrhoid, liver, gallbladder and lymph nodes 9–180 d after COVID-19
Hany et al. <sup>29</sup>	NM	N	NM	Gastric and gallbladder tissues 274–380 d after COVID-19
Miura et al. <sup>30</sup>	✓	N	No	Adenoid tonsil, adenoid tissue, nasal cytobrush and nasal wash from children with no documented COVID-19 or upper airway infection in the month before collection
Xu et al. <sup>37</sup>	✓	NM	No	Child adenoid and tonsil tissue up to 303 d after COVID-19
Peluso et al. <sup>24</sup>	✓	NM	✓	Colorectal lamina propria tissue 158–676 d after COVID-19
Yao et al. <sup>25</sup>	✓	S, N	✓	Fungiform papillae tongue tissue 6–63 weeks after COVID-19
<b>Tissue (autopsy)</b>				
Stein et al. <sup>31</sup>	✓	N	NM	Dozens of human body and brain tissue types at least 31 d and up to 230 d after COVID-19
Roden et al. <sup>32</sup>	✓	NM	NM	Lung tissue up to 174 d after COVID-19
Rendiero et al. <sup>26</sup>	NM	S	NM	Lung tissue up to 359 d after COVID-19
<b>Stool</b>				
Natarajan et al. <sup>115</sup>	✓	NM	✓	Stool up to 230 d after COVID-19
Yonker et al. <sup>84</sup>	✓	S, N	✓	RNA in stool of children with MIS-C 13–62 d after COVID-19, S and N protein in plasma
Jin et al. <sup>116</sup>	✓	S	NM	Neonatal stool in infants born to mothers whose COVID-19 symptoms resolved more than 10 weeks before delivery
<b>Blood</b>				
Schultheiß et al. <sup>40</sup>	NM	S1	✓	Plasma at a median time of 8 months after COVID-19
Swank et al. <sup>41</sup>	NM	S, S1, N	✓	Plasma up to 12 months after COVID-19
Peluso et al. <sup>44</sup>	NM	S1, N	✓	Plasma neuron-derived EVs 35–84 d after COVID-19
Peluso et al. <sup>42</sup>	NM	S1, S, N	✓	Plasma up to 16 months after COVID-19
Craddock et al. <sup>45</sup>	✓	S	✓	Spike linked to EVs in samples obtained at least 8–12 weeks (up to 1 year) after COVID-19
Tejerina et al. <sup>117</sup>	✓	NM	✓	Plasma at a median time of 55 d after COVID-19 (also found in stool/urine at the same median time point)

✓, identified; No, not present; NM, not measured; S and S1, spike protein.

in the same sample. Whether the viral RNA and EV-associated spike protein originate from the same tissue or cellular source and why they are detected as separate entities remains unclear. Overall, EVs may facilitate the transport of SARS-CoV-2 proteins from tissue reservoir sites into the circulation.

The identification of SARS-CoV-2 protein in PASC plasma up to 16 months after COVID-19 suggests that some patients with PASC may harbor replicating virus. However, thus far, levels of protein detected differ widely among studies, suggesting that the size and/or activity of any SARS-CoV-2 reservoirs may vary among patients with PASC. Failure to detect SARS-CoV-2 protein in the plasma of some patients with PASC could be interpreted to mean absence of a SARS-CoV-2 reservoir. However, such a result could also indicate a reservoir in tissues or sites where viral protein may be less likely to reach the circulation at the level of detection of current assays. In addition, protein could be bound by antibodies, preventing recognition by some assays. Moreover, SARS-CoV-2 protein might also be captured and potentially persist inside neutrophil extracellular traps (NETs) or host immune cells such as macrophages and thus also fail to be detected via analyses of plasma alone.

Variability in detection of different viral proteins in PASC plasma could also reflect differences in SARS-CoV-2 translational activity. For example, Swank et al. reported multiple PASC cases in which spike

protein was identified in plasma of the same individual at some time points but not others<sup>41</sup>. These findings suggest it may be possible that SARS-CoV-2 in a reservoir could have periods of inactivity and resume protein production and/or replication at other times such as when immune control is altered. Such a phenomenon is in line with the fluctuating symptoms reported by many patients with PASC. A study of survivors with post-Ebola syndrome suggests that the activity of persistent viral RNA in reservoir sites can change over time. Adaken et al. reported declines and subsequent rises—or a ‘decay–stimulation–decay’ pattern—in neutralizing antibody levels in the plasma of Ebola virus disease survivors<sup>46</sup>. This periodic neutralizing antibody resurgence likely corresponds to periods of more active replication in Ebola virus reservoir sites, followed by periods of relative inactivity. Similar waves of recurrent immune activation consistent with periodic increases in immune stimulation by viral proteins have also been documented in measles<sup>47</sup>. Further interrogating such relationships in PASC is warranted.

Additional research is needed to better understand the role of persistent SARS-CoV-2 protein or RNA in causing ongoing symptoms. For example, it will be necessary to interrogate how location of infection and viral dissemination within the host, transcriptional/translational activity of SARS-CoV-2 RNA, virus genomic evolution, human genomic variants, HLA haplotypes and other variables are connected



to differences in host innate and adaptive responses and/or predispose people to persistence of viral protein or RNA. Moreover, interrogating factors underlying the detection of viral protein in convalescent individuals without PASC—albeit at lower levels than in PASC participants—will be of considerable interest. Such studies should help determine the relationships between viral persistence, immune responses and development of PASC in only some individuals following SARS-CoV-2 infection.

## Adaptive immunity and PASC SARS-CoV-2 reservoir

The immune response can act as a sensitive indicator of virus persistence. T cell differentiation is strongly influenced by antigen exposure, even if at low levels and chronic<sup>48,49</sup>. T cells can detect a single HLA–peptide complex and the process of antigen recognition triggers phenotypic and transcriptional changes among responsive T cells<sup>50–53</sup>. T cells also often become more sensitive to other environmental signals because of their activation<sup>49</sup>. Therefore, distinct patterns of T cell differentiation can provide clues to infer the presence of a SARS-CoV-2 reservoir. For example, Vibholm et al. analyzed SARS-CoV-2-specific CD8<sup>+</sup> T cell responses using a dextramer stain for nine different CD8<sup>+</sup> T cell epitopes<sup>54</sup>. Individuals who harbored SARS-CoV-2 pharyngeal RNA 2 weeks after COVID had increased breadth and magnitude of SARS-CoV-2-specific CD8<sup>+</sup> T cell responses.

Multiple studies have identified SARS-CoV-2-specific T cells or altered responses to SARS-CoV-2 peptide pool stimulation in at least a subset of PASC participants, consistent with viral or antigen persistence<sup>55</sup>. Littlefield et al. quantified inflammatory markers and SARS-CoV-2-specific T cells in PASC versus convalescent participants<sup>56</sup>. The circulating frequencies of functionally responsive CD4<sup>+</sup> and CD8<sup>+</sup> T cells, identified by measuring cytokine production in response to stimulation with SARS-CoV-2 peptide pools, were 6- to 105-fold higher in individuals with pulmonary PASC. These patients also displayed elevated plasma C-reactive protein and interleukin (IL)-6 compared with control participants. Similar findings were reported in a study of individuals with neurological PASC who exhibited more-pronounced cellular and humoral immune responses targeting the SARS-CoV-2 N protein than those of convalescent control patients<sup>57</sup>.

Other teams have identified markers of persisting immune activation and/or T cell exhaustion consistent with ongoing stimulation by SARS-CoV-2 antigens and/or a skewed inflammatory environment in PASC participants. For example, Yin et al. found that PASC participants harbored significantly higher SARS-CoV-2 antibodies, and elevated frequencies of central memory T cells, follicular helper T (T<sub>FH</sub>) cells and regulatory T cells in blood<sup>58</sup>. Production of IL-6 by SARS-CoV-2 spike-specific CD4<sup>+</sup> T cells was detected in some PASC participants, suggesting a potential link to inflammatory responses. SARS-CoV-2-specific CD8<sup>+</sup> T cells from PASC participants also more frequently expressed PD-1 and CTLA-4: markers of recent T cell activation and/or exhaustion. Indeed, Klein et al. found that elevated frequencies of CD8<sup>+</sup> T cells and CD4<sup>+</sup> T cells from PASC participants expressed both PD-1 and Tim-3 (ref. 59), consistent with chronic antigen stimulation and presence of exhausted T cells. Elevated anti-spike antibody responses in plasma were also identified in individuals with PASC, suggestive of persistent spike protein driving elevation in the humoral responses.

Some adaptive immune responses in PASC blood are consistent with a SARS-CoV-2 reservoir in mucosal tissue. In the Yin et al. study, CD4<sup>+</sup> T cells in patients with PASC preferentially expressed the chemokine receptors CCR6, CXCR4 and CXCR5, which can direct T cells to inflammatory sites, including the lungs in some settings<sup>58</sup>. Moreover, Cruz et al. documented persistent immunological alterations in patients with PASC, including redistribution of CD8<sup>+</sup> T cells expressing the mucosal homing  $\beta 7$  integrin and higher levels of plasma IgA against SARS-CoV-2 S and N proteins, suggesting possible mucosal involvement<sup>60</sup>.

Interrogating cells involved in, or derived from, germinal center (GC) responses including virus-specific B cells, antibody-secreting cells (ASCs) and CD4<sup>+</sup> T<sub>FH</sub> cells could also provide insights about SARS-CoV-2 antigen or RNA persistence in PASC. In other settings, for example in studies of viral RNA persistence after alphavirus or persistent measles virus infection, a characteristic feature is either local tissue residence of virus-specific ASCs<sup>61,62</sup> and/or ongoing GC reactions and production of ASCs<sup>63</sup>. Ongoing stimulation of immune responses by viral RNA long after acute disease has resolved results in the continued appearance of ASCs and circulating T<sub>FH</sub> cells in peripheral blood and maturation of plasma antibody avidity<sup>63</sup>. Persistent influenza virus antigen in lung-draining lymph nodes is also thought to drive GC responses that can last for months<sup>64–66</sup>. Overall, these data suggest that GC B cells and/or T<sub>FH</sub> cells might be used as biosensors to infer the persistence of viral antigens<sup>49</sup>.

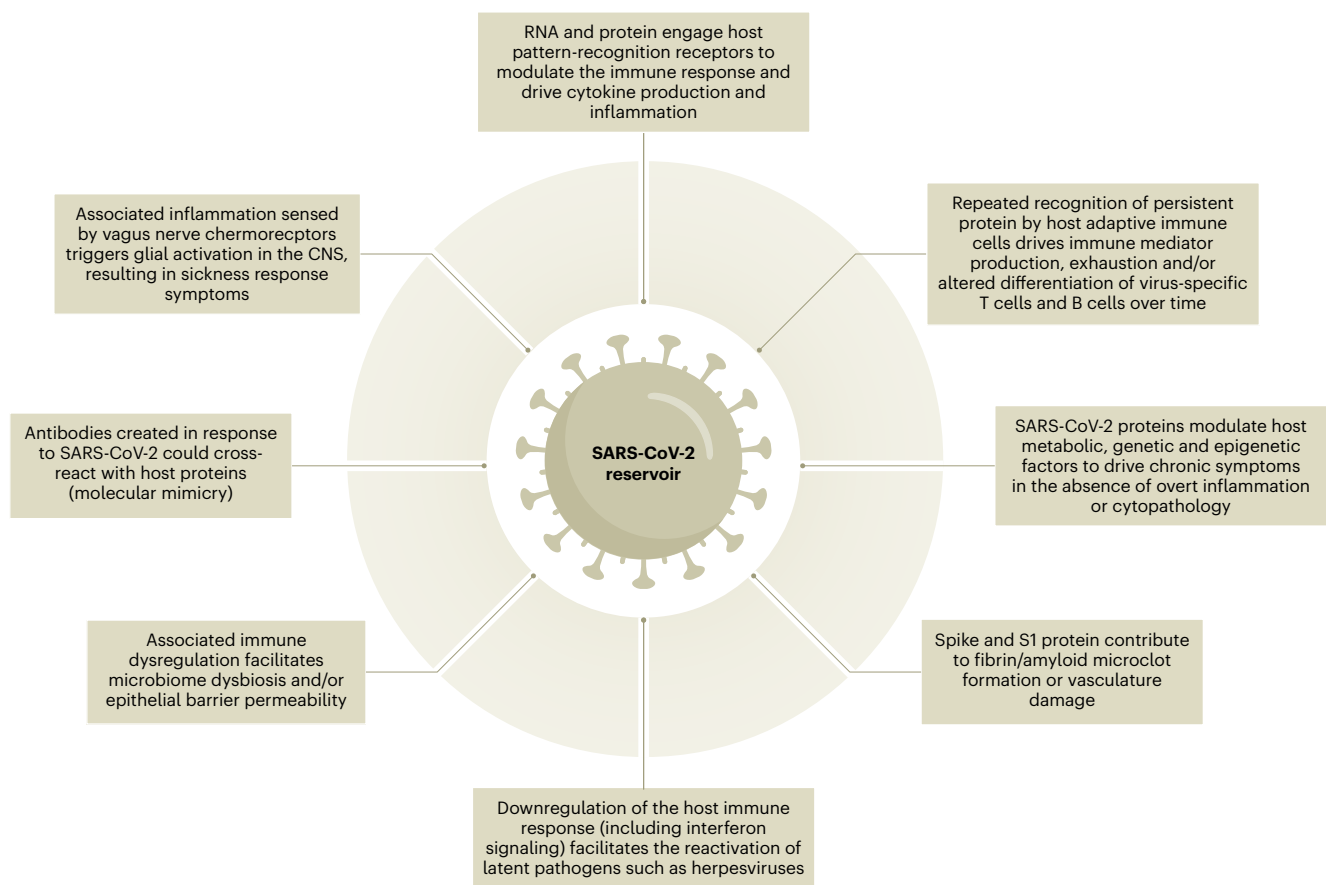
There is some evidence that SARS-CoV-2 can persist in lymphoid tissues where GCs are located<sup>30</sup>. While not performed in PASC (symptoms were not measured as part of the study), Xu et al. identified persistent expansion of GC and antiviral lymphocyte populations associated with interferon (IFN)- $\gamma$ -type responses in pharyngeal lymphoid tissues (tonsil and adenoid) collected via surgery from non-vaccinated COVID-19-convalescent children<sup>37</sup>. SARS-CoV-2 nucleocapsid RNA was identified in 15 of 22 tonsil, and 7 of 9 adenoid samples, despite negative nasopharyngeal swab RT–PCR results at the time of surgery. In four cases where tissue was examined, the last positive nasopharyngeal swab RT–PCR was <100–300 d before surgery. Viral RNA copies significantly correlated with the percentages of S1-positive and receptor-binding-domain-positive B cells among GC B cells in tonsil tissue, suggesting that SARS-CoV-2 antigen persistence contributed to the prolonged lymphoid and GC responses. How such persisting GC responses relate to PASC remains to be explored.

## Mechanisms of disease

The persistence of SARS-CoV-2 RNA and/or proteins in PASC reservoir sites could drive disease via several non-mutually exclusive mechanisms (Fig. 1). Persistent viral RNA and/or protein might engage host pattern-recognition receptors, provoking cytokine production and inflammation. Repeated recognition of persistent protein by host adaptive immune cells could result in effector activity, exhaustion and/or altered differentiation of virus-specific T cells and B cells over time, any of which could contribute to tissue damage or pathology.

Active SARS-CoV-2 replication, or persistence or production of viral proteins and/or RNA, could also be directly cytopathic. As many cells express the receptors necessary for virus entry, direct damage could occur in a wide array of tissues or organ systems. Infection of neurons or nerves, for example, could lead to direct damage in the central or peripheral nervous systems. However, SARS-CoV-2 RNA or protein could drive PASC pathology via mechanisms that do not result in overt inflammation or tissue cytopathology. Multiple SARS-CoV-2 proteins can downregulate the host innate immune response<sup>67</sup>, suggesting that local responses may be disabled rather than activated. SARS-CoV-2 proteins are also capable of modulating host metabolic, genetic and epigenetic factors<sup>68</sup> to dysregulate the activity of host signaling pathways in a manner that could drive a range of chronic symptoms in the absence of overt cytopathology.

A SARS-CoV-2 reservoir in PASC could also contribute to coagulation and vasculature-related issues. Pretorius et al. identified fibrin/amyloid microclots resistant to fibrinolysis (indicative of hypercoagulation) in PASC platelet-poor plasma<sup>69</sup>. They also showed that addition of the SARS-CoV-2 S1 protein to healthy platelet-poor plasma resulted in structural changes to fibrinogen (including resistance to trypsinization) similar to the fibrin deposits identified in the microclots<sup>70</sup>. Another study demonstrated that the SARS-CoV-2 spike protein can bind to fibrinogen and induce structurally abnormal blood clots with heightened proinflammatory activity<sup>71</sup>. Thus,



**Fig. 1 | Mechanisms by which a SARS-CoV-2 reservoir may contribute to PASC.** Adapted with permission from K. Boils.

SARS-CoV-2 S1 or spike protein in PASC plasma may directly contribute to microclot formation, localized tissue fibrin accumulation and related vascular issues. In fact, SARS-CoV-2 spike protein has been identified inside COVID-19 thrombi<sup>72</sup>, suggesting it might be possible for microclots to entrap viral proteins. Entrapment of SARS-CoV-2 protein inside microclots could represent another reason that SARS-CoV-2 protein might not be easily identified in the plasma of patients with PASC who have a viral reservoir. Persistence of spike antigen in plasma could also trigger formation of proinflammatory immune complexes and/or NETs that can contribute to clotting processes. For example, one study found that addition of spike protein to convalescent COVID-19 plasma containing SARS-CoV-2 antibodies led to the formation of antigen–antibody immune complexes that induced significantly increased NETosis compared with convalescent COVID-19 plasma alone<sup>73</sup>.

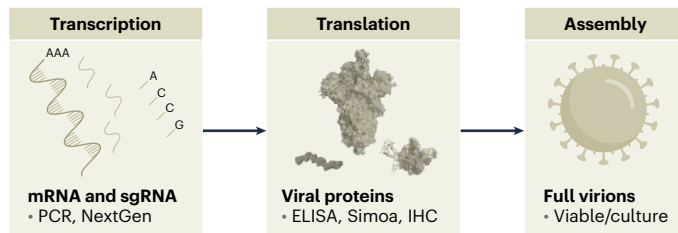
Dysregulation of the immune response by SARS-CoV-2 reservoir could also facilitate the reactivation of latent infections. Expression of SARS-CoV-2 proteins that downregulate host interferon signaling<sup>74,75</sup>—signaling central to successful control of persisting viral infections—may be particularly detrimental in this regard. Indeed, reactivation of latent herpesvirus, such as Epstein–Barr virus (EBV), has been associated with PASC<sup>59,76–78</sup>. However, the relationship between herpesvirus reactivation in PASC and potential persistence of SARS-CoV-2 in the same individual/cohort remains incompletely understood.

### SARS-CoV-2 reservoir may contribute to microbiome imbalance

RNA virus infections correlate with microbiome alterations and the outgrowth of opportunistic microorganisms<sup>79</sup>. These observations suggest that dysregulation of the host immune response

by SARS-CoV-2 in tissue could negatively impact host microbiome diversity or activity in the same or distant body sites. Because microbiome-derived metabolites are major regulators of host immune, metabolic and hormonal signaling, microbiome imbalance or dysbiosis can drive a range of pathological processes<sup>79,80</sup>. Microbiome activity also contributes to priming of the immune system and the production of compounds that disable pathogens. Thus, it is possible that microbiome dysbiosis could predispose people to altered acquisition or clearance of SARS-CoV-2 infection. For example, women with vaginal microbiome dysbiosis are more likely to acquire human immunodeficiency virus<sup>81</sup>. Microbiome dysbiosis has been reported in PASC<sup>82</sup> but thus far has not been studied in concert with SARS-CoV-2 persistence in the same body site.

SARS-CoV-2 reservoir and/or microbiome dysbiosis in the gastrointestinal tract, oral cavity or other body sites can be accompanied by low-grade local inflammation that promotes dysfunction or breakdown of epithelial barriers. This increased epithelial barrier permeability facilitates the translocation of SARS-CoV-2 proteins or microbial products into the bloodstream, where they can drive or sustain inflammatory processes<sup>83</sup>. For example, Yonker et al. found that children with MIS-C harbored SARS-CoV-2 RNA in stool weeks after initial infection<sup>84</sup>. This RNA detection was accompanied by SARS-CoV-2 spike protein in plasma and significantly increased release of zonulin—a biomarker of intestinal permeability<sup>85,86</sup>. These findings suggest that in MIS-C, prolonged persistence of SARS-CoV-2 in the gastrointestinal tract drives zonulin-instigated permeability of the mucosal barrier, with subsequent increased trafficking of SARS-CoV-2 protein from the gut into the bloodstream, leading to hyperinflammation<sup>87</sup>. A similar phenomenon might occur in people with PASC.



**Fig. 2 | Components of SARS-CoV-2 measured in persistence studies.**

IHC, immunohistochemistry; sgRNA, subgenomic RNA. Adapted and reproduced with permission from K. Boils.

## SARS-CoV-2 reservoir and cross-reactive autoimmunity

SARS-CoV-2 can induce antibody responses that are cross-reactive with host proteins, with at least one mechanism being molecular mimicry (sequence homology between viral antigens and host receptors or proteins). For example, Kreye et al. identified high-affinity SARS-CoV-2-neutralizing antibodies that cross-reacted with mammalian heart, gut, lung, kidney and brain self antigens<sup>88</sup>. Autoreactive T cells and antibodies can be induced during acute infection, but also may be continually promoted by a persistent SARS-CoV-2 reservoir. Recent evidence shows that EBV is an example of a persistent virus that can drive molecular mimicry-based autoimmunity. In an analysis of multiple sclerosis cerebrospinal fluid, Lanz et al. demonstrated molecular mimicry between the EBV protein EBNA1 and the CNS protein GlialCAM<sup>89</sup>. Given the connections between EBV and PASC mentioned above, these observations further highlight the need for additional studies on the relationship between the two viruses.

## SARS-CoV-2 reservoir may alter vagus nerve signaling

A SARS-CoV-2 reservoir could also contribute to nonspecific PASC symptoms including fatigue, trouble concentrating, muscle and joint pain, sleep dysfunction, anxiety, depression, loss of appetite and autonomic dysfunction<sup>90</sup>. These symptoms overlap with the sickness response (called ‘sickness behavior’ in animal models) that reflects the subjective and behavioral component of innate immunity and is largely mediated by signaling of the vagus nerve<sup>90,91</sup>.

Tens of thousands of afferent vagus nerve branches innervate all major trunk organs with chemoreceptor terminals, which collectively act as a sensitive and diffuse neuroimmune sensory organ for the CNS. These branches can detect highly localized paracrine immune signaling such as cytokine activation even in the absence of a systemic circulating immune response<sup>90</sup>, triggering glial activation and neuroinflammation on the brain side of the blood–brain barrier and the sickness response. The persistence of a SARS-CoV-2 reservoir in body sites densely innervated by the vagus nerve (for example, gut, lung and bronchial tubes)—or direct infection of the vagus nerve<sup>92</sup> as has been shown in autopsy studies<sup>93,94</sup>—might activate localized paracrine signaling, leading to ongoing sickness response symptoms in infected individuals.

## SARS-CoV-2 reservoir and neurodegenerative sequelae

Direct infiltration and persistence of SARS-CoV-2 in the CNS is also a potential driver of neuroinflammation and/or cognitive, neurological and psychiatric symptoms in individuals with PASC. SARS-CoV-2 neuroinvasion potential has been shown in organoid and animal models<sup>27,95</sup> and in several autopsy studies that prioritized short post-mortem intervals<sup>31,94</sup>. Such neuroinvasion may be relevant to the apparent post-acute COVID-19 sequela of increased Alzheimer’s disease incidence. Wang et al. found that older adults (age ≥65 years) had a significantly increased risk for a new Alzheimer’s disease diagnosis

within 360 d after acute COVID-19 disease<sup>96</sup>. A separate autopsy study demonstrated increased amyloid- $\beta$  (A $\beta$ ) plaque deposition in brain tissue obtained from severely ill individuals hospitalized with COVID-19 who were younger than 60 years old<sup>97</sup>. Alzheimer’s disease A $\beta$  ‘plaque’ protein has been shown to function as an antimicrobial peptide that forms as part of the host innate immune response toward pathogens capable of infecting brain tissue. In a series of in vitro and animal experiments, Eimer et al. demonstrated A $\beta$  accumulation via extracellular trap agglutination in response to bacteria, fungi and viruses (including herpes simplex virus type 1)<sup>98–100</sup>. Thus, SARS-CoV-2 persistence in the CNS—or CNS reactivation of other pathogens such as herpesviruses after COVID-19—might also contribute to activation of an evolutionarily conserved role for A $\beta$  as an antimicrobial peptide, increasing both short-term and long-term risk for Alzheimer’s disease.

## Major areas of investigation

Many aspects of a SARS-CoV-2 reservoir in PASC and the impact of viral activity on related biological factors require further study. More research is needed to understand if SARS-CoV-2 RNA identified in PASC tissue samples months after acute COVID-19 is actively transcribed, translated, replicated and/or is infectious. SARS-CoV-2 protein detection could indicate replicating virus and/or transcribable viral RNA (Fig. 2). However, the persistence of both SARS-CoV-2 protein and RNA after acute COVID-19 may differ by cell type or anatomical location due to differences in the local immune environment and/or the lifespan or turnover of infected cells. For example, lymph node B cell follicles can harbor antigen for extended periods of time as antigen–antibody complexes on follicular dendritic cells<sup>101</sup>. However, long-term persistence of SARS-CoV-2 protein in the absence of replicating virus is much less likely in cell types that experience rapid turnover—such as intestinal epithelial cells. Autopsy studies and additional tissue biopsy studies, which together offer unparalleled access to broad tissue types, must be performed in PASC so that these potentially distinct features of SARS-CoV-2 reservoir sites can be better delineated. Such efforts would be greatly facilitated by a PASC registry combined with a coordinated autopsy research program.

Viral culture is the gold standard for identification of infectious SARS-CoV-2 but has not been successful in post-COVID-19 samples<sup>33,38</sup>. However, viral growth from such samples is challenging for many reasons including susceptibility of the cell line to different strains, presence of neutralizing antibody in the sample and limiting amounts of material available. In addition, multiple biological mechanisms can suppress the production of infectious virions to facilitate the survival of infected cells despite viral RNA persistence. For example, viral mutations can accumulate that decrease virion assembly or decrease RNA synthesis, while host cells engage antiviral immune responses that facilitate infected cell survival<sup>102</sup>. Indeed, acquisition of viral mutations is a well-established mechanism that facilitates the persistence of certain RNA viruses including coronaviruses<sup>103</sup>.

Further study is also required to better understand if SARS-CoV-2 RNA and/or protein persistence in certain PASC tissues or body fluids may differ based on viral variant (for example, delta versus omicron), and the unique manner by which different viral variants may evade the host immune response. For example, SARS-CoV-2 can downregulate major histocompatibility complex class I expression to evade CD8<sup>+</sup> T cell recognition<sup>104</sup>, with more effective evasion by omicron subvariants<sup>105</sup>. Suboptimal antiviral host responses typified by early induction of non-neutralizing antibodies and anti-inflammatory posttranslational modification of immunoglobulin Fc regions might also facilitate SARS-CoV-2 persistence in PASC.

The questions in Box 1 highlight major research areas of opportunity that should provide further clarity on the role of a SARS-CoV-2 reservoir in the PASC disease process. Diverse approaches and methodologies must be used to address these central research questions. These include autopsy studies, imaging studies, tissue biopsy studies,



**BOX 1**

## Major areas of opportunity for research into a SARS-CoV-2 reservoir in PASC

- Which PASC cell and tissue types harbor SARS-CoV-2 RNA or protein? Is there a preference for persistence in certain cell or tissue types?
- Is SARS-CoV-2 RNA identified in PASC samples transcriptionally active, translating, replicating or infectious?
- Is the presence of a SARS-CoV-2 reservoir sufficient to drive PASC symptoms? Are SARS-CoV-2 RNA and proteins also identified in samples collected from post-COVID-19 individuals without PASC? If yes, what factors differentiate SARS-CoV-2 persistence in PASC from persistence in asymptomatic individuals?
- Do particular classes of symptoms tend to be driven by the location of the reservoir—for example, dyspnea from a lung reservoir or gastrointestinal symptoms from a gut reservoir?
- Do measurements of SARS-CoV-2 protein or antibody responses in body fluids correlate with SARS-CoV-2 persistence in tissue?
- Can the transcriptional program of circulating immune cells be used as a biosensor of SARS-CoV-2 persistence in tissue? Does T cell exhaustion correlate with SARS-CoV-2 persistence in PASC?
- Are neutralizing antibody responses qualitatively different in patients with PASC?
- By what mechanisms can SARS-CoV-2 evade immune detection? Do such mechanisms differ by cell or tissue type, or by viral variant? Do viral mutations and selection contribute to persistence?
- Can the spike protein travel via EVs into the bloodstream?
- Does a SARS-CoV-2 reservoir or protein contribute to fibrin/amyloid microclotting, platelet activation or related vasculature issues in PASC?
- Does a SARS-CoV-2 reservoir in PASC correlate with the reactivation of other pathogens such as herpesviruses?
- Does a SARS-CoV-2 reservoir in PASC correlate with changes in human endogenous retrovirus activity?
- Can a SARS-CoV-2 reservoir alter the local transcriptome or epigenome?
- Does a SARS-CoV-2 reservoir in PASC correlate with the disruption of microbiome composition or activity? If so, is disruption a cause or consequence of PASC?
- Is a SARS-CoV-2 reservoir associated with host epithelial barrier breakdown in PASC? Does this facilitate the translocation viral protein or bacterial/fungal organisms into blood?
- Can SARS-CoV-2 persistence or the reactivation of other latent pathogens lead to cross-reactive antibody responses in PASC blood or tissue?

use of ultrasensitive assays to identify viral protein, use of immune cells as biosensors of SARS-CoV-2 persistence and other methods (Supplementary Note).

### Biomarker and therapeutic targets for PASC clinical trials

Research on SARS-CoV-2 reservoir and related biological factors in PASC will enable the identification of (1) biomarkers for improved PASC diagnosis, (2) biomarkers that serve as primary outcome measures for

PASC clinical trials and (3) therapeutic candidates for PASC clinical trials. Potential therapeutics for the treatment of SARS-CoV-2 reservoir in PASC include direct-acting and host-directed antivirals and immunomodulators that can boost the immune response (for example, interferons and monoclonal antibodies). Early case reports suggest that SARS-CoV-2 antivirals may benefit certain patients with PASC<sup>106</sup>. For example, a patient with PASC reported resolution of symptoms and a return to pre-COVID-19 health function after a 5-day course of the SARS-CoV-2 antiviral nirmatrelvir-ritonavir (Paxlovid)<sup>107</sup>. Such anecdotal cases highlight the need for rigorous clinical trials designed to address this hypothesis, and multiple double-blind, randomized clinical trials of direct-acting antivirals such as Paxlovid for the proposed treatment of SARS-CoV-2 reservoir in PASC are planned or underway (see ClinicalTrials.gov identifiers [NCT05576662](#), [NCT0566809](#), [NCT05823896](#) and [NCT05595369](#)).

However, some forms of antiviral treatment may only show benefit if SARS-CoV-2 is actively replicating and spreading from cell to cell. It is also possible that a single course of approved SARS-CoV-2 antivirals is not adequate to fully address viral persistence in all relevant PASC cases. Indeed, even for acute infection viral rebound after treatment due to incomplete viral clearance is well documented. Therefore, treatment of a SARS-CoV-2 reservoir in PASC may require longer dosing periods to achieve maximum efficacy. Moreover, combining more than one antiviral both increases efficacy and reduces the risk of resistance. For example, Schultz et al. demonstrated that combining pyrimidine biosynthesis inhibitors with antiviral nucleoside analogs synergistically inhibits SARS-CoV-2 infection *in vitro* and *in vivo* against emerging strains of SARS-CoV-2 during acute respiratory infection<sup>108</sup>. Regimens for other RNA viruses capable of persistence (for example, human immunodeficiency virus and hepatitis C virus) require multiple drugs for robust long-term benefit.

Treatment with antivirals or combinations of antivirals and immune-modulating agents during acute COVID-19 may also prevent PASC by decreasing or eliminating virus that might otherwise persist in a reservoir. Acute COVID-19 antiviral clinical trials should consequently be designed to capture the impact of treatment on PASC development. For example, Xie et al. estimated the effect of the antiviral nirmatrelvir (versus control) on covariate-standardized hazard ratio and absolute risk reduction of a prespecified panel of 12 post-acute COVID-19 outcomes after 90 d (ref. 109). They found that in individuals with SARS-CoV-2 infection with at least one risk factor for progression to severe COVID-19 illness, nirmatrelvir treatment within 5 d of a positive COVID-19 test was associated with reduced risk of PASC regardless of history of prior infection and vaccination status.

Research findings should also inform how therapies against SARS-CoV-2 might best be combined with other treatment modalities in PASC. These therapies could include herpesvirus antivirals, microbiome-based therapeutics, anticoagulant medications and vagus nerve stimulation. Some of these therapeutics may be tailored to the site of the reservoir. For example, treatment of an individual with MIS-C with larazotide to restore gut epithelial barrier permeability resulted in a decrease in plasma SARS-CoV-2 spike antigen levels and inflammatory markers, accompanied by clinical improvement<sup>84,110</sup>. Similar approaches aimed at restoring normal gut barrier permeability might also be used in PASC in concert with antivirals or immunomodulators.

### Conclusion

SARS-CoV-2 reservoir may drive inflammatory, coagulation, microbiome, neuroimmune and other abnormalities in PASC. Future research should focus on determining if SARS-CoV-2 persistence varies by cell type or body site, by viral variant, and should further delineate mechanisms by which SARS-CoV-2 evades immune detection or elimination to persist in human tissue. Factors that differentiate SARS-CoV-2 persistence in PASC from persistence in asymptomatic individuals should be explored. More research is needed to understand if SARS-CoV-2

RNA in PASC reservoir sites is being actively transcribed, translated, replicated and/or is infectious. A PASC autopsy program and additional PASC tissue biopsy studies are required to best address these central research questions.

More broadly, the study of SARS-CoV-2 reservoir and related biological factors in PASC may inform the identification of disease mechanisms, biomarkers and therapeutics for other chronic conditions increasingly tied to persistent viral infection. These diseases include myalgic encephalomyelitis/chronic fatigue syndrome<sup>111</sup>, Alzheimer's disease<sup>99</sup>, and autoimmune diseases such as multiple sclerosis<sup>89,112</sup> and systemic lupus erythematosus<sup>113</sup>. While a growing body of evidence connects the pathogenesis of these conditions to the activity of persistent DNA viruses, it is possible that RNA viruses previously studied primarily for their ability to drive acute illness could also contribute to disease in a chronic capacity. Synergistic approaches developed to characterize a SARS-CoV-2 reservoir in PASC could be rapidly incorporated into the study of chronic RNA virus activity in these related conditions to inform a deeper understanding of shared biological mechanisms.

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## Competing interests

A.D.P. has received consulting fees from Enanta Pharmaceuticals outside the submitted work. S.A. has received honoraria for lectures and educational events from Gilead, AbbVie, MSD and Biogen, and reports grants from Gilead and AbbVie. E.W.E. has received grant support/research funding from the US National Institutes of Health and Veterans Administration, is an unfunded investigator with baricitinib on COVID-19 studies funded by Eli Lilly, and has lectured at events related to sedation in the intensive care unit sponsored by Pfizer. M.F. reports a relationship with Mars that includes board membership. L.N.G. reports receiving grants from Pfizer and advisory fees from UnitedHealthcare. D.E.G. is a member of the scientific advisory committees for GSK, Merck and Takeda Pharmaceuticals. T.J.H. consults for Roche and received grant support from Merck. A.I. cofounded and consults for RIGImmune, Xanadu Bio and PanV; consults for Paratus Sciences, InvisiShield Technologies; and is a member of the Board of Directors of Roche Holding. M.J.P. has

received consulting fees from Gilead Sciences and AstraZeneca, outside the submitted work. E.P. founded Biocode Technologies and holds a patent for detection of microclots in blood samples. E.J.W. is a member of the Parker Institute for Cancer Immunotherapy, which supports cancer immunology research in the laboratory of E.J.W. E.J.W. is an advisor for Danger Bio, Janssen, New Limit, Marengo, Pluto Immunotherapeutics, Related Sciences, Santa Ana Bio, SyntheKine and Surface Oncology. E.J.W. is a founder of and holds stock in Surface Oncology, Danger Bio and Arsenal Biosciences.

## Additional information

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