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

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Summary/abstract

Millions of patients 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 SARS-CoV-2 virus 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 protein 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.

Introduction

A significant subset of individuals infected with the SARS-CoV-2 virus 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) [1]. 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[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[3].

Common PASC symptoms include fatigue, flu-like symptoms, autonomic dysfunction, trouble with memory or concentration, and post-exertional malaise (PEM) [4]. However, more than 200 PASC symptoms have been documented and symptom presentation can differ from patient to patient [5 6]. In addition, many individuals with PASC report symptoms of fluctuating severity or a relapsing/remitting nature[7]. PASC can occur in children, with an incidence of up to 25% of cases in earlier COVID-19 waves[8], and more recent reports suggesting that roughly 6% of children infected with SARS-CoV-2 meet PASC criteria.[9] 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 US alone [10]. 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 a significant proportion of 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 PASC patient 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 (EBOV) [11–13], Zika virus (ZIKV)[14], enteroviruses [15,16], and measles[17 18] has been identified in tissue obtained months or years after initial infection. In multiple instances these viral reservoirs have been shown capable of driving chronic disease [19 20]. In the case of Ebola virus disease (EBV), new outbreaks of disease have been sparked by individuals carrying persistent EBOV years after acute illness [21 22], and there are multiple reports of sexual transmission of ZIKV many months after recovery from acute disease[23].

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 [24–26 27 28 29 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 days and up to 230 days after COVID-19 symptom onset [31]. 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 CNS including the cervical spinal cord, brainstem, and olfactory nerve. In one individual who died 230 days after mild COVID-19, SARS-CoV-2 RNA was identified in multiple anatomical sites, including several brain regions. Subgenomic (Sg)RNA - a potential marker of recent viral replication - was identified in tissues post-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 days after COVID-19 onset [32].

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[31 33]. 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 [34 35 36]. Immune responses against SARS-CoV-2 RNA and protein, including those indicative of persistence, can also be localized to tissue and not necessarily apparent in the blood from the same individuals. [37]

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 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 [38]. Despite mild acute infections, 70% of subjects harbored SARS-CoV-2 RNA in intestinal mucosal tissue and 52% had nucleocapsid 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 did associate with PASC symptoms. Another study identified SARS-CoV-2 RNA and nucleocapsid protein (N) in the skin, appendix, and breast tissue of two individuals who exhibited PASC symptoms 163 and 426 days after acute COVID-19 [39]. SARS-CoV-2 RNA or protein was also detected in olfactory mucosa samples 110-196 days after symptom onset in 3 patients with negative nasopharyngeal swab RT-PCR, but ongoing anosmia [27].

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 controls [40]. Using an optimized ultra-sensitive single-molecule array (Simoa) method, Swank *et al.* identified either spike, S1, or nucleocapsid (N) protein in ~65% of plasma samples collected from PASC patients several months after SARS-CoV-2 infection [41]. Spike was detected most often: in 60% of PASC participants up to 12 months post COVID-19 onset, with no spike detected in COVID-19 convalescent controls. Viral protein was detected at more than one timepoint in all 12 of the 37 PASC cases for whom the team had obtained longitudinal samples. Additional Simoa analyses in another post-acute cohort[7] 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 timepoint up to 16 months post-COVID [42] with most of these data obtained before subjects had received any SARS-CoV-2 vaccine, a potential confounder in such analyses[43]. 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 ≥ 9 symptoms). Notably, a subset of convalescent controls who reported full recovery (18%) also had detectable viral protein in plasma.

In addition to persisting as soluble protein 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 PASC individuals versus convalescent controls [44]. Craddock *et al.* identified spike protein in the plasma of 64% of PASC patients and 29% of convalescent controls [45]. 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 controls, yet only PASC study participants harbored both spike protein and viral RNA 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 post-COVID suggests that some PASC individuals 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 PASC patients**.** Failure to detect SARS-CoV-2 protein in the plasma of some PASC patients 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 macrophage 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 timepoints but not others [41]. 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 PASC individuals. 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 (nAb) in the plasma of EVD survivors [46]. This periodic nAb resurgence likely corresponds to periods of more active replication in EBOV 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 [47]. 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 to persistence of viral protein or RNA. Moreover, interrogating factors underlying the detection of viral protein in convalescent subjects 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 low-level and chronic [48] [49]. T cells can detect a single HLA/peptide complex and the process of antigen recognition triggers phenotypic and transcriptional changes among responsive T cells [50–52] [53]. T cells also often become more sensitive to other environmental signals because of their activation[49]. 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 $CD8^+$ T cell responses using a dextramer stain for nine different $CD8^+$ T cell epitopes [54]. Individuals who harbored SARS-CoV-2 pharyngeal RNA two weeks post-COVID had increased breadth and magnitude of SARS-CoV-2-specific $CD8^+$ 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 [55]. Littlefield *et al.* quantified inflammatory markers and SARS-CoV-2-specific T cells in PASC versus convalescent participants[56]. The circulating frequencies of functionally responsive $CD4^+$ and $CD8^+$ 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 IL-6 compared to controls. 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 compared to convalescent controls[57].

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 patients. For example, Yin *et al.* found that PASC patients harbored significantly higher SARS-CoV-2 antibodies, and elevated frequencies of Tcm, Tfh, and Treg in blood [58]. Production of IL-6 by SARS-CoV-2 spike-specific CD4+ T cells was detected in some PASC patients, suggesting a potential link to inflammatory responses. SARS-CoV-2-specific $CD8^+$ T cells from PASC patients 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 $CD8^+$ T cells and $CD4^+$ T cells from PASC patients expressed both PD-1 and Tim-3 [59], consistent with

chronic antigen stimulation and presence of exhausted T cells (Tex). 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, $CD4^+$ T cells in PASC individuals preferentially expressed the CCR6, CXCR4, and CXCR5 chemokine receptors that can direct T cells to inflammatory sites, including the lungs in some settings [58]. Moreover, Cruz *et al.* documented persistent immunological alterations in PASC patients, including redistribution of $CD8^+$ T cells expressing the mucosal homing β7 Integrin and higher levels of plasma IgA against SARS-CoV-2 S and N proteins, suggesting possible mucosal involvement [60].

Interrogating cells involved in or derived from germinal center (GC) responses including virus-specific B cells, antibody secreting cells (ASC), and T follicular helper (Tfh) $CD4^+$ T 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 antibody-secreting cells (ASCs)[61,62] and/or ongoing GC reactions and production of ASCs[63]. Ongoing stimulation of immune responses by viral RNA long after acute disease has resolved results in the continued appearance of ASCs and circulating Tfh cells in peripheral blood and maturation of plasma antibody avidity [63]. Persistent influenza virus antigen in lung-draining lymph nodes is also thought to drive GC responses that can last for months [64 65 66]. Overall, these data suggest that GC B cells and/or Tfh cells might be used as biosensors to infer the persistent viral antigens [49].

There is some evidence that SARS-CoV-2 can persist in lymphoid tissues where GC are located [30]. 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)-γ-type responses in pharyngeal lymphoid tissues (tonsil and adenoid) collected via surgery from non-vaccinated COVID-19-convalescent children [37]. SARS-CoV-2 nucleocapsid RNA was identified in 15 out of 22 tonsil, and 7 out of 9 adenoid samples, despite negative nasopharyngeal swab RT-PCRs at the time of surgery. In 4 cases where tissue was examined, the last positive nasopharyngeal swab RT-PCR had been ~100-300 days before surgery. Viral RNA copies significantly correlated with the percentages of $S1^+RBD^+$ 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 (Figure 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 [67], 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 [68] 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 (PPP) [69]. They also showed that addition of the SARS-CoV-2 S1 protein to healthy PPP resulted in structural changes to fibrinogen (including resistance to trypsinization) similar to the fibrin deposits identified in the microclots [70]. Another study demonstrated that the SARS-CoV-2 spike protein can bind to fibrinogen and induce structurally abnormal blood clots with heightened proinflammatory activity [71]. Thus, 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[72], 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 PASC patients with 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 significant NETosis compared with convalescent COVID-19 plasma alone[73].

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 [74 75] – 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[76 59 77 78]. However, the relationship between herpesvirus reactivation in PASC and potential persistence of SARS-CoV-2 in the same patient/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 microbes [79]. These observations suggest that dysregulation of the host immune responses 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 [79 80]. 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 to an altered SARS-CoV-2 infection. For example, women with vaginal microbiome dysbiosis are more likely to acquire HIV [81]. Microbiome dysbiosis has been reported in PASC [82], 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 [83]. For example, Yonker *et al.* found that children with MIS-C harbored SARS-CoV-2 RNA in stool weeks after initial infection [84]. This RNA detection was accompanied by SARS-CoV-2 spike protein in plasma and significantly increased release of zonulin - a biomarker of intestinal permeability [85,86]. 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 [87]. A similar phenomenon might occur in patients with PASC.

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 [88]. 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 EBV protein nuclear antigen 1 (EBNA1) and the central nervous system protein glial cell adhesion molecule (GlialCAM)[89]. 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 non-specific PASC symptoms including fatigue, trouble concentrating, muscle and joint pain, sleep dysfunction, anxiety, depression, loss of

appetite, and autonomic dysfunction [90]. 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[90 91]. 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 central nervous system. These branches can detect highly localized paracrine immune signaling such as cytokine activation even in the absence of a systemic circulating immune response[90], 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 (e.g., gut, lung, bronchial tubes, etc.) - or direct infection of the vagus nerve[92] as has been shown in autopsy studies [93 94]- 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[9527] and in several autopsy studies that prioritized short postmortem intervals [31,94]. Such neuroinvasion may be relevant to the apparent post-acute COVID-19 sequela of increased Alzheimer's disease (AD) incidence. Wang *et al.* found that older adults (age ≥65 years) had a significantly increased risk for a new AD diagnosis within 360 days after acute COVID-19 [96]. A separate autopsy study demonstrated increased amyloid beta (Aβ) plaque deposition in brain tissue obtained from severely ill, hospitalized COVID-19 patients younger than 60 years old[97]. AD amyloid beta "plaques" can function as an antimicrobial peptide that forms as part of the host innate immune response towards pathogens in brain tissue. In a series of *in vitro* and animal experiments, Eimer *et al*. demonstrated Aβ accumulation via extracellular trap agglutination in response to bacteria, fungi, and viruses (including HSV-1)[98 99 100]. Thus, SARS-CoV-2 persistence in the CNS - or CNS reactivation of other pathogens such as herpesviruses post-COVID - might also contribute to activation of an evolutionarily conserved role for Aβ as an antimicrobial peptide, increasing both short and long-term risk for AD.

Major areas of investigation

Many aspects of SARS-CoV-2 persistence 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 (Figure 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 [101]. 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 samples [38 33]. 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[102]. Indeed, acquisition of viral mutations is a well-established mechanism that facilitates the persistence of certain RNA viruses including coronaviruses [103].

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 (e.g., 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 (MHC) class I expression to evade $CD8^+$ T cell recognition[104], with more effective evasion by omicron subvariants [105]. Suboptimal antiviral host responses typified by early induction of non-neutralizing antibodies and anti-inflammatory post-translational 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 employed to address these central research questions. These include autopsy studies, imaging studies, tissue biopsy studies, use of ultrasensitive assays to identify viral protein, use of immune cells as biosensors of SARS-CoV-2 persistence, and other methods (see Supplementary Note).

Biomarker and therapeutic targets for PASC clinical trials

Research on SARS-CoV-2 reservoir and related biological factors in PASC will enable identification of 1) biomarkers for improved PASC diagnosis; 2) biomarkers that serve as primary outcome measures for PASC clinical trials; 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 (e.g., interferons and monoclonals antibodies). Early case reports suggest that SARS-CoV-2 antivirals may benefit certain PASC individuals [106]. For example, a PASC patient 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) [107]. 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 NCT05576662, NCT0566809, 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, Cherry *et al*. demonstrated that combining pyrimidine biosynthesis inhibitors with antiviral nucleoside analogues synergistically inhibits SARS-CoV-2 infection *in vitro* and *in vivo* against emerging strains of SARS-CoV-2 during acute respiratory infection [108]. Regimens for other RNA viruses capable of persistence (e.g., HIV, HCV) 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 days[109]. They found that in individuals with SARS-CoV-2 infection with at least 1 risk factor for progression to severe COVID-19 illness, nirmatrelvir treatment within five days 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 a MIS-C patient 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 [84110]. Similar approaches aimed at restoring normal gut barrier permeability might also be employed 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 a SARS-CoV-2 evades immune detection or elimination to persist in patient 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 include myalgic encephalomyelitis/chronic fatigue syndrome (ME/CFS) [111], Alzheimer's disease [99], autoimmune diseases such as multiple sclerosis [89] [112]and systemic lupus erythematosus [113]. 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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Author contributions statement

A.D.P., M.B.V., S.A., K.B., B.P.B, M.B., S.C, D. S.C., H.E.D., C.L.D., S.D., W.E., E.W.E., A.F., M.F., L.N.G., D.G., T.J.H., A.I., D.I., M.L., S.M., M.P., M.J.P., E.P., D.A.P, D.P., R.H.S., G.S.T., R.E.T., H.F.V., L.M.Y., and E.J.W. contributed to writing and editing. A.D.P. wrote the initial draft of the manuscript and conceived of the figures and tables. E.J.W. supervised and edited writing of the manuscript. M.B.V. edited and improved the manuscript and conceived of Figure 2.

Competing interest statement

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 NIH/VA, is an unfunded Investigator with Baricitinib on COVID-19 studies funded by Eli Lily, and has lectured at events related to sedation in the ICU 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.G. is a member of scientific advisory committees for GSK, Merck and Takeda Pharmaceuticals. T.J.H. consults for Roche and received grant support from Merck. A.I. co-founded 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 Ltd. M.J.P. has received consulting fees from Gilead Sciences and AstraZeneca, outside the submitted work. R.P. founded Biocode Technologies and hold 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 his laboratory. 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. The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Table 1:

Identification of SARS-CoV-2 RNA and protein post COVID-19

	RNA	Protein	PASC symptoms	Location
Tissue - biopsy				
Goh *et al*[39].	√	S, N	√	Appendix, skin, and breast tissues 163 and 426 days post-COVID-19
Zollner *et al*[38].	√	N	√	Gut mucosa/epithelium tissue ~7 months post-COVID-19
deMelo *et al*[27].	√	N	√	Olfactory neuroepithelium tissue 110–196 days post-COVID-19
Gaebler *et al*.[33]	√	N	No	Intestinal tissue ~4 months post-COVID-19
Cheung *et al*[114].	√	S, N	NM	Colon, appendix, ileum, hemorrhoid, liver, gallbladder, lymph node 9-180 days post-COVID-19
Hany *et al*[29].	NM	N	NM	Gastric and gallbladder tissues 274-380 days post-COVID-19
Miura *et al*[30].	√	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*[37].	√	NM	No	Child adenoid and tonsil tissue up to 303 days post-COVID-19
Tissue - autopsy				
Stein *et al*[31].	√	N	NM	Dozens of human body and brain tissue types at least 31 days and up to 230 days post-COVID19
Roden *et al*[32].	√	NM	NM	Lung tissue up to 174 days post-COVID-19
Bussani *et al*[24].	√	S, N	NM	Bronchial cartilage chondrocytes, para bronchial gland epithelial cells, vascular pericytes, endothelial cells average 105.5 days post-COVID-19
Böszörményi *et al*[25].	√	NM	NM	Macaque extrapulmonary tissues including heart, respiratory tract, surrounding lymph nodes, salivary gland, and conjunctiva 5-6 weeks post-COVID-19
Rendiero *et al*[115].	NM	S	NM	Lung tissue up to 359 days post-COVID-19
Stool				
Natarajan *et al*[116].	√	NM	√	Stool up to 230 days post-COVID-19
Yonker *et al*[84].	√	S, N	√	RNA in stool of children with MIS-C 13–62 days post-COVID-19, S and N protein in plasma
Jin *et al*[117].	√	S	NM	Neonatal stool in infants born to mothers whose COVID-19 symptoms resolved more than 10 weeks prior to delivery
Blood				
Schultheiß *et al*[40].	NM	S1	√	Plasma at a median time of 8 months post-COVID-19
Swank *et al*[41].	NM	S, S1, N	√	Plasma up to 12 months post-COVID-19
Peluso *et al*[44].	NM	S1, N	√	Plasma neuron-derived extracellular vesicles 35-84 days post-COVID-19
Peluso *et al*[42].	NM	S1, S, N	√	Plasma up to 16 months post-COVID-19
Craddock *et al*[45].	√	S	√	Spike linked to extracellular vesicles in samples obtained at least 8-12 weeks (up to 1 year) post-COVID-19

Tejerina *et al*[118].	√	NM	√	Plasma at a median time of 55 days post-COVID-19 (also found in stool/urine at the same median timepoint)	

Box 1:

- 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 patients 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, i.e., dyspnea from a lung reservoir, GI 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 extracellular vesicles into the bloodstream?
- Does SARS-CoV-2 reservoir or protein contribute to fibrin/amyloid microclotting, platelet activation, or related vasculature issues in PASC?
- Does SARS-CoV-2 reservoir in PASC correlate with the reactivation of other pathogens such as herpesviruses?
- Does SARS-CoV-2 reservoir in PASC correlate with changes in Human Endogenous Retrovirus (HERV) activity?
- Can a SARS-CoV-2 reservoir alter the local transcriptome or epigenome?
- Does 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 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?

Figure legends

Figure 1: Mechanisms by which a SARS-CoV-2 reservoir may contribute to PASC. BioRender licensed software was not used to create the figure.

Figure 2: Components of SARS-CoV-2 measured in persistence studies. BioRender licensed software was not used to create the figure.

Table 1: √ = identified, No - not present, NM =not measured, S= spike protein, N= nucleocapsid protein

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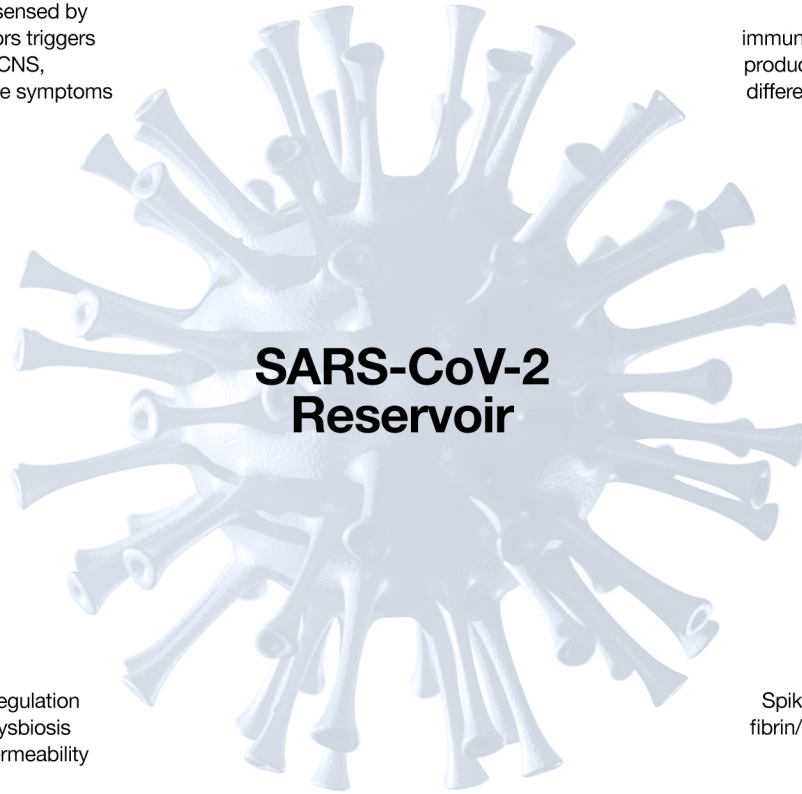
Mechanisms by which SARS-CoV-2 reservoir may contribute to PASC

RNA and protein engage host pattern-recognition receptors to modulate the immune response and drive cytokine production and inflammation

Associated inflammation sensed by vagus nerve chemoreceptors triggers glial activation in the CNS, resulting in sickness response symptoms

Repeated recognition of persistent protein by host adaptive immune cells drives immune mediator production, exhaustion and/or altered differentiation of virus-specific T cells and B cells over time.



Antibodies created in response to SARS-CoV-2 could cross react with host proteins (molecular mimicry)

SARS-CoV-2 proteins modulate host metabolic, genetic, and epigenetic factors to drive chronic symptoms in the absence of overt inflammation or cytopathology

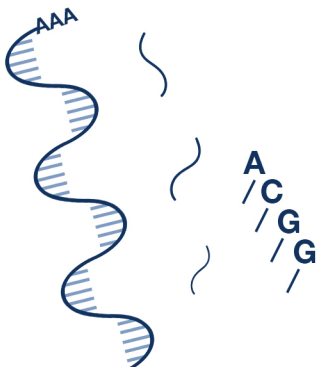
Associated immune dysregulation facilitates microbiome dysbiosis and/or epithelial barrier permeability

Spike or S1 protein contributes to fibrin/amyloid microclot formation or vasculature damage

Downregulation of the host immune response (including interferon signaling) facilitates the reactivation of latent pathogens such as herpesviruses

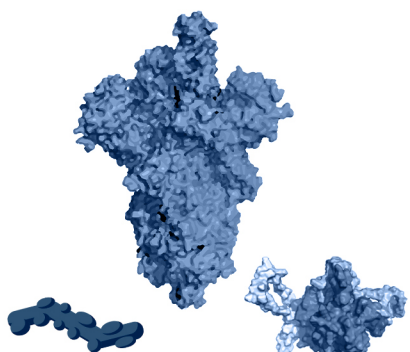
Components of SARS-CoV-2 measured in persistence studies

Transcription → **Translation** → **Assembly**



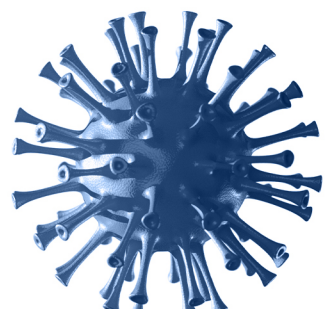
mRNA **sgRNA**

PCR, NextGen



viral proteins

ELISA, Simoa, ICH



full virions

viable/culture

Diverse approaches and methodologies can be used in the study of SARS-CoV-2 reservoir

Autopsy Studies

Strengths

- Can identify SARS-CoV-2 RNA and protein in tissues that cannot be obtained safely via biopsy, including from the CNS
- Viral genome sequencing can identify SARS-CoV-2 mutations associated with persistence in certain anatomical locations
- Tissue cytopathology near identified RNA and protein can be assessed

Weaknesses

- Short postmortem interval is necessary for optimal tissue preservation
- Perimortem changes can alter the tissue's transcriptional landscape, meaning tissue is not optimized for transcriptome-based approaches that capture host immune & gene expression

Imaging Studies

Strengths

- Can identify SARS-CoV-2 spike protein and T cell activity in tissue locations in living patients that otherwise cannot be accessed via biopsy
- SARS-CoV-2 spike protein and T cell activity in a wide range of tissue sites can be measured simultaneously

Weaknesses

- High cost of analysis and imaging scanners only available in limited locations
- Radioligands are limited by penetration and specificity

Biopsy & Surgical Sample Studies

Strengths

- Samples can be preserved immediately and optimally for both genomic and protein analyses
- Allows for optimal use of sequencing technologies to characterize host immune and gene expression changes near identified SARS-CoV-2 RNA or protein (e.g., spatial transcriptomics)

Weaknesses

- Only certain tissue types can be safely obtained via biopsy, and tissue sample size must be small

Ultrasensitive Protein & Antibody Detection in Fuids

Strengths

- Analysis can be performed on body fluids (e.g., blood, saliva) which can be collected non-invasively and at routine study visits

Weaknesses

- Protein from SARS-CoV-2 reservoir in certain tissues or CNS sites may not enter body fluids or could be bound by antibodies. This could prevent recognition by relevant immunoassays
- In some cases it is known that circulating markers do not accurately reflect local responses (e.g., cytokines)

Adaptive Immune Cells as Biomarkers of Persistence

Strengths

- The adaptive immune response can act as a sensitive indicator of virus persistence, with single-molecule detection possible at the level of cognate viral epitopes displayed on the infected cell surface

Weaknesses

- Expensive and requires a large amounts of sample to isolate specific cell types

Organoid & Animal Studies

Strengths

- Can be directly infected and used to explore mechanisms of persistence, including how viral variants and mutations can contribute to the survival of infected cells

Weaknesses

- Lack of homology between animal model pathways and human pathways must be considered
- Culture and organoid models are incomplete biological systems