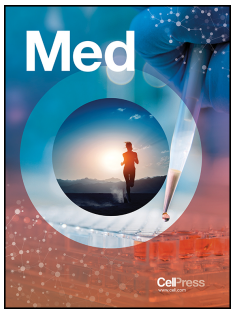
Journal Pre-proof



Gastrointestinal symptoms and fecal shedding of SARS-CoV-2 RNA suggest prolonged gastrointestinal infection

Aravind Natarajan, Soumaya Zlitni, Erin F. Brooks, Summer E. Vance, Alex Dahlen, Haley Hedlin, Ryan M. Park, Alvin Han, Danica T. Schmidtke, Renu Verma, Karen B. Jacobson, Julie Parsonnet, Hector F. Bonilla, Upinder Singh, Benjamin A. Pinsky, Jason R. Andrews, Prasanna Jagannathan, Ami S. Bhatt

PII: S2666-6340(22)00167-2

DOI: https://doi.org/10.1016/j.medj.2022.04.001

Reference: MEDJ 195

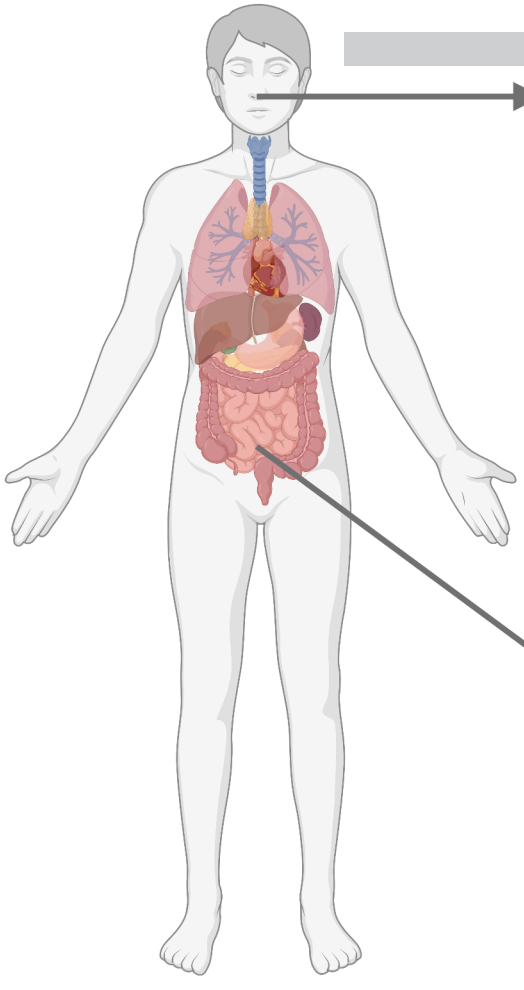
To appear in: *Med*

Received Date: 27 January 2022

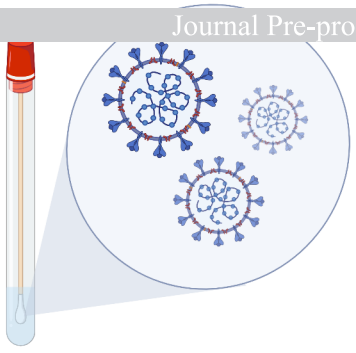
Revised Date: 1 March 2022

Accepted Date: 5 April 2022

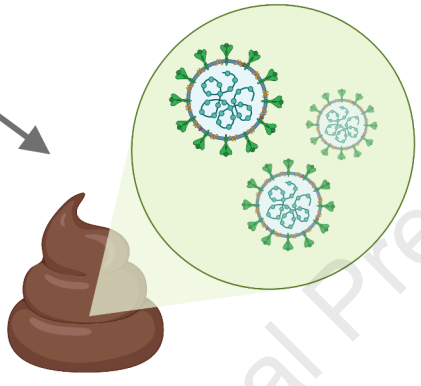
Please cite this article as: Natarajan, A., Zlitni, S., Brooks, E.F., Vance, S.E., Dahlen, A., Hedlin, H., Park, R.M., Han, A., Schmidtke, D.T., Verma, R., Jacobson, K.B., Parsonnet, J., Bonilla, H.F., Singh, U., Pinsky, B.A., Andrews, J.R., Jagannathan, P., Bhatt, A.S., Gastrointestinal symptoms and fecal shedding of SARS-CoV-2 RNA suggest prolonged gastrointestinal infection, *Med* (2022), doi: https://doi.org/10.1016/j.medj.2022.04.001.



Patient with COVID-19



SARS-CoV-2 RNA in respiratory samples

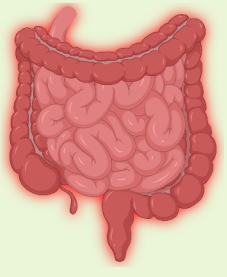


SARS-CoV-2 RNA in fecal samples

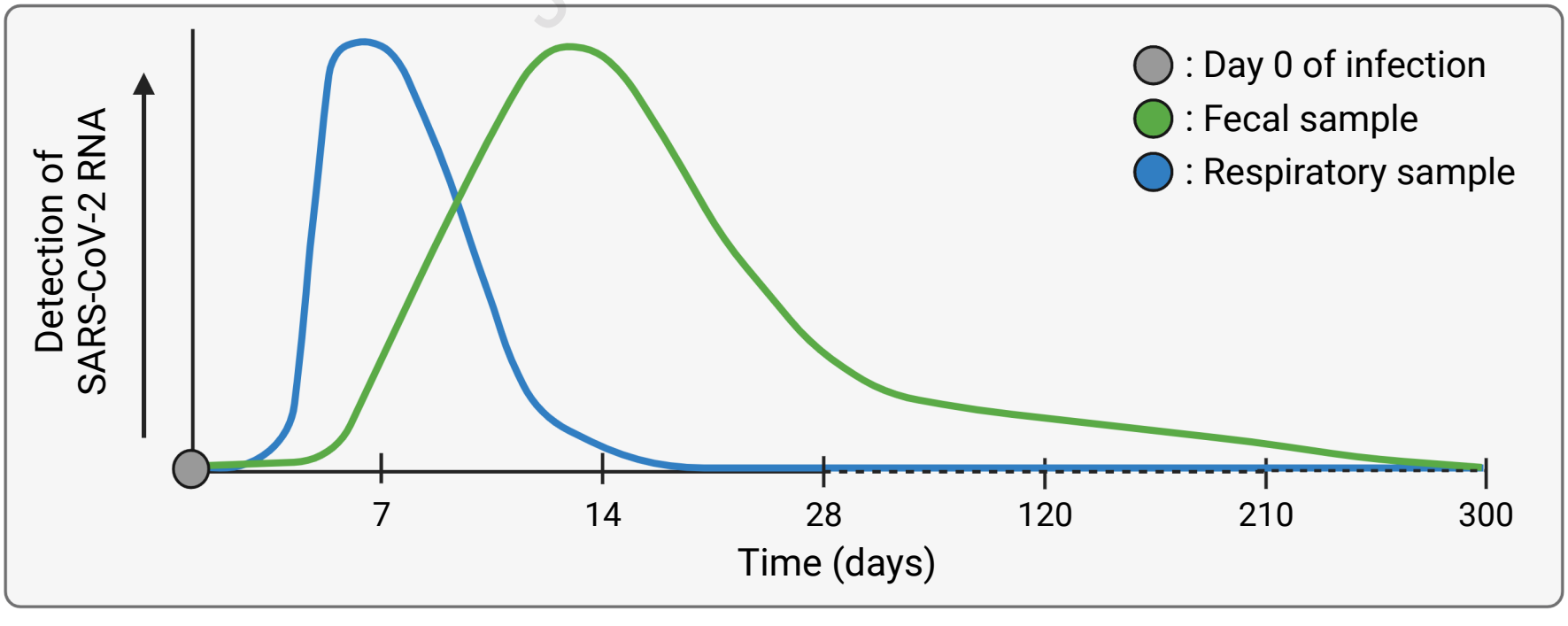
Longest recorded fecal shedding

210 Days

Fecal shedding is associated with GI symptoms



GI tract is a potential site of SARS-CoV-2 infection



Title

Gastrointestinal symptoms and fecal shedding of SARS-CoV-2 RNA suggest prolonged gastrointestinal infection

Authors

Aravind Natarajan[1,2,*], Soumaya Zlitni[1,2,*], Erin F. Brooks[2,*], Summer E. Vance[2,*], Alex Dahlen[3], Haley Hedlin[3], Ryan M. Park, Alvin Han[4], Danica T. Schmidtke[4], Renu Verma[5], Karen B. Jacobson[5], Julie Parsonnet[6,7], Hector F. Bonilla[6], Upinder Singh[5], Benjamin A. Pinsky[5,8], Jason R. Andrews[5], Prasanna Jagannathan[4,6], Ami S. Bhatt^[1,2]

Affiliations

[1] Department of Genetics, Stanford University, Stanford, CA, USA.

[2] Department of Medicine (Hematology, Blood and Marrow Transplantation), Stanford University, Stanford, CA, USA.

[3] Quantitative Science Unit, Stanford University, Stanford, CA, USA.

[4] Department of Microbiology and Immunology, Stanford University, Stanford, CA, USA.

[5] Department of Medicine (Infectious Diseases and Geographic Medicine), Stanford University, Stanford, CA, USA.

[6] Department of Medicine (Infectious Diseases), Stanford University, Stanford, CA, USA.

[7] Department of Medicine (Epidemiology and Population Health), Stanford University, Stanford, CA, USA.

[8] Department of Pathology, Stanford University, Stanford, CA, USA

*These authors contributed equally

^Lead contact

Contact info

^ Ami S. Bhatt, 269 Campus Dr, CCSR 1155b, Stanford University, Palo Alto, CA 94305. Tel: (650) 498-4438; Email: asbhatt@stanford.edu.

ORCID information

Aravind Natarajan	0000-0003-2180-7842
Soumaya Zlitni	0000-0003-2228-8343
Erin F. Brooks	0000-0002-5834-0679
Summer E. Vance	0000-0002-1347-4729
Alex Dahlen	0000-0002-7911-6203
Haley Hedlin	0000-0002-0757-7959
Ryan M. Park	0000-0002-7426-2068
Alvin Han	0000-0003-4525-0866
Danica T. Schmidtke	0000-0001-9509-6188
Renu Verma	0000-0003-1277-8824
Karen B. Jacobson	0000-0003-3050-4595
Julie Parsonnet	0000-0001-7342-5366
Hector F. Bonilla	
Upinder Singh	
Benjamin A. Pinsky	0000-0001-8751-4810
Jason Andrews	0000-0002-5967-251X

Prasanna Jagannathan 0000-0001-6305-758X

Ami S. Bhatt 0000-0001-8099-2975

Abstract

Background: COVID-19 manifests with respiratory, systemic, and gastrointestinal (GI) symptoms.[1,2] SARS-CoV-2 RNA is detected in respiratory and fecal samples, and recent reports demonstrate viral replication in both the lung and intestinal tissue.[3–5] Although much is known about early fecal RNA shedding, little is known about the long term shedding, especially in those with mild COVID-19. Furthermore, most reports of fecal RNA shedding do not correlate these findings with GI symptoms.[6]

Methods: We analyze the dynamics of fecal RNA shedding up to 10 months after COVID-19 diagnosis in 113 individuals with mild to moderate disease. We also correlate shedding with disease symptoms.

Findings: Fecal SARS-CoV-2 RNA is detected in 49.2% [95% Confidence interval = 38.2%-60.3%] of participants within the first week after diagnosis. Whereas there was no ongoing oropharyngeal SARS-CoV-2 RNA shedding in subjects at and after 4 months, 12.7% [8.5%-18.4%] of participants continued to shed SARS-CoV-2 RNA in the feces at 4 months after diagnosis and 3.8% [2.0%-7.3%] shed at 7 months. Finally, we find that GI symptoms (abdominal pain, nausea, vomiting) are associated with fecal shedding of SARS-CoV-2 RNA.

Conclusions: The extended presence of viral RNA in feces, but not respiratory samples, along with the association of fecal viral RNA shedding with GI symptoms suggest that SARS-CoV-2

infects the GI tract, and that this infection can be prolonged in a subset of individuals with COVID-19.

Funding: This research was supported by a Stanford ChemH-IMA grant, fellowships from the AACR and NSF and NIH R01-AI148623, R01-AI143757, and UL1TR003142.

Keywords

COVID-19, SARS-CoV-2, Fecal RNA, Gastrointestinal infection, viral shedding

Introduction

COVID-19 is a disease with protean manifestations, ranging from respiratory to gastrointestinal to systemic. While the primary site of infection of SARS-CoV-2 is the respiratory tract, the presence of symptoms affecting other organ systems (e.g. abdominal pain, nausea, arthralgia), coupled with in vitro evidence of SARS-CoV-2 infectivity in a variety of other tissues, suggests that SARS-CoV-2 infection can extend beyond the respiratory system. Meta-analyses of studies that focus on hospitalized individuals with COVID-19 estimate the pooled incidence of gastrointestinal (GI) symptoms such as nausea, vomiting, and diarrhea to be between 11 and 18%.[1,3,4,7–9] Additionally, within this moderate to severe disease group, SARS-CoV-2 RNA has been detected in 40 to 85% of fecal samples (reviewed in Brooks EF and Bhatt AS[10]), indicating that SARS-CoV-2 viral RNA is found in feces nearly as frequently as in respiratory secretions.[11] Patients with moderate to severe COVID-19 have been well studied; by contrast, much less is known about the clearance of SARS-CoV-2 RNA in the feces of patients with mild to moderate disease, despite the fact that they make up ~81% of those who contract COVID-19.[12,13] Furthermore, most studies are cross-sectional, and the few reported longitudinal studies have focused on the early time points after diagnosis. Thus, a comprehensive understanding of the dynamics of fecal clearance of SARS-CoV-2 RNA in individuals with mild to moderate COVID-19 is both of crucial importance and lacking.

Interestingly, in the few studies that have investigated longitudinal fecal samples, prolonged fecal shedding of SARS-CoV-2 RNA can occur even after viral RNA clearance in respiratory samples. Indeed, in one notable pediatric case, viral RNA shedding extended beyond 70 days after disease onset[9]. If SARS-CoV-2 RNA shedding in the feces is indicative of a GI

infection, this suggests that SARS-CoV-2 infection of the GI tract can continue after clearance from the respiratory tract.

While the presence of SARS-CoV-2 RNA in feces is well established, whether live, infectious SARS-CoV2 is commonly shed in stool remains an outstanding question (reviewed in Guo M et. al.[14]). Five studies have reported isolating infectious SARS-CoV-2 from stool samples collected from participants with severe COVID-19,[15–19] while others have reported being unable to isolate infectious virions from stool.[20,21] Therefore it remains unclear whether the presence of infectious virions of SARS-CoV-2 in the stool is a rare or common phenomenon. However, there is mounting evidence of possible SARS-CoV-2 infection of the GI tract. Specifically, presence of SARS-CoV-2 RNA,[5,22–24] protein antigen[22,25] and virions[5,24,26] in GI biopsies all point to a potential infection of the GI tract. Additional supportive evidence of a GI infection by SARS-CoV-2 is the presence of a gut immune response[27] as well as inflammation measured by markers such as fecal calprotectin[28,29] in individuals with COVID-19. Finally, *in vitro* experiments reveal that SARS-CoV-2 is able to successfully infect enteroid models of the gut[30–32] and intestinal cell lines.[33] This phenomenon of possible GI tract involvement is not surprising as bovine coronavirus (BCoV) and human enteric coronavirus (HECoV-4408), both of the same genus as SARS-CoV-2 (*Betacoronaviruses*), can infect respiratory and GI tissues.[34] Taken together, these data indicate that the GI tract may be an important site of SARS-CoV-2 infection.[34]

SARS-CoV-2 presence in the GI tract has additional relevance to patient health. The GI tract is a highly immunoactive tissue, and SARS-CoV-2 antigens in this body site may hone a humoral immune response against variants of the SARS-CoV-2 virus.[22] Further, prolonged

presence of SARS-CoV-2 in the GI tissue may also have an impact on the hitherto mysterious phenomenon of post-acute sequelae of SARS-CoV-2 infection (PASC) or 'Long COVID', where individuals suffer from an unusual constellation of symptoms even after recovery from the respiratory SARS-CoV-2 infection.[35] Taken together, it is critical that we understand whether or not the GI tract is infected, and the dynamics of the infection in this tissue - both from the standpoint of the acute infection as well as the long term sequelae of COVID-19.

Here, we sought to better define the features of SARS-CoV-2 presence in the GI tract and its relevance for short- and long-term human health. We leveraged longitudinal fecal and respiratory samples from individuals enrolled in a randomized controlled study of Peg-interferon λ vs. a placebo control for the treatment of mild to moderate COVID-19 (n = 120).[36] While the intervention did not shorten the duration of oropharyngeal shedding of SARS-CoV-2 (primary outcome) or symptoms (secondary outcome), the study provided a rich, prospectively collected dataset from which to evaluate fecal shedding dynamics and its relation to GI symptoms.

Using fecal samples collected at regular intervals from the time of COVID-19 diagnosis to 10 months after diagnosis, we compared fecal viral RNA shedding to the presence of GI and other symptoms and found that it is positively correlated with GI symptoms. This constitutes the largest longitudinal analysis of paired fecal viral RNA shedding and disease symptomatology data in individuals with mild to moderate COVID-19, and reveals important information about the pathophysiology of the disease.

Results

Description of study participants and sample collection

The Peginterferon Lambda-1a (IFN-λ) clinical trial (NCT04331899) enrolled 120 participants with mild to moderate COVID-19 between 25 April and 17 July 2020.[36] Of these, 113 participants collected at least one stool sample at one of the six predefined stool collection time points. These collection time points centered around days 3 (range = 0 - 7 days), 14 (8 - 21), 28 (22 - 35), 120 (75 - 165), 210 (166 - 255) and 300 (> 255 days) post-enrollment **(Fig. 1A)**. Out of these 113 participants, 86 provided samples for at least three time points (summarized in Data S**1**).

We originally started collecting stool samples in the OMNIGene GUT collection tube (OG), which is extensively used in gut microbiome studies.[37] Parallel work from our group[11] and one other group[38] optimized and benchmarked stool collection and processing methods for the detection of fecal SARS-CoV-2 RNA; our group found that the Zymo DNA/RNA shield fecal collection tube (ZY) performs better than OG in viral RNA preservation. Therefore, starting 14 May 2020, study participants were asked to provide samples in both the OG and ZY kits. Overall, a total of 326 samples were collected in the OG kit, and 347 in the ZY kit (sample collection compliance is summarized in Data S**1,** Related to Figures 1, 2 and 3, and STAR Methods, additional data and analysis that informs methods and conclusions in the study). In addition to these stool samples, oropharyngeal (OP) swabs were obtained daily during the initial part of the study, and at each study visit on days 120, 210, and 300; blood samples were drawn at days 0, 5, 14, 28, 120, 210, and 300 **(Fig. 1A)**. Clinical specimens were paired with self-reported symptom data collected through questionnaires administered on the day of enrollment, then daily

from day 1 through 28, and on days 120, 210, and 300. Additionally, symptoms experienced in the three weeks preceding study enrollment were surveyed on the day of enrollment. Finally, long term follow-up questionnaires on days 120, 210, and 300 collected symptoms occurring in the seven days leading up to the appointment.

Among the participants who returned at least one stool sample, the median age was 36 years (IQR = 29 - 51 years), 46 (41%) were female, and 72 (65%) were Hispanic **(Fig. 1B, Table 1)**. We describe the overall cohort, as well as two subsets: those reporting gastrointestinal (GI) symptoms (n = 54, 49%) at the first time point, and those reporting no GI symptoms (i.e. exclusively respiratory symptoms or no symptoms at all) at this time point. Participants with GI symptoms at baseline are more likely to also experience a constellation of other symptoms, including myalgias (participants with GI symptoms = 78%, without GI symptoms = 30%, standard difference = -1.09), chills (59%, 21%, -0.84), decreased smell (63%, 30%, -0.7), headache (70%, 42%, -0.59) and joint pain (46%, 19%, -0.6). A comparison of those with and without GI symptoms, in terms of age, sex, ethnicity, and clinical measures at enrollment including temperature, blood oxygen saturation, white blood cell count, blood alanine aminotransferase (ALT) concentration, and SARS-CoV-2 IgG seropositivity reveal no large differences and are presented in **Table 1**.

Longitudinal dynamics of SARS-CoV-2 RNA in stool

673 stool samples collected from 113 participants over a period of 10 months were processed as per a recently optimized and benchmarked protocol[11] outlined in the methods and summarized in Figure S**1**. Briefly, RNA was extracted from each of these stool samples, and

assayed for four target genes in the SARS-CoV-2 genomic RNA (gRNA) encoding the Envelope protein (E), nucleocapsid protein (N1 and N2) and RNA-dependent RNA polymerase (RdRP) in technical duplicate, using RT-qPCR. We also assayed 278 of the 673 RNA samples derived predominantly from samples collected in the first month of the study for the N1 and E gene using multiplexed droplet digital PCR (ddPCR) assays, since ddPCR is more robust to the presence of inhibitors of PCR than RT-qPCR[39]. We found the measurement of the N1 and E genes using ddPCR to be concordant with one another (Figure S**2)**, and thus assayed the remainder of the samples (n = 395) only for the N1 gene. In total, 5,384 RT-qPCR assays and 951 ddPCR assays measuring the concentration of fecal SARS-CoV-2 gRNA were carried out. This dataset was then analyzed as summarized in the STAR methods. SARS-CoV-2 viral RNA concentrations estimated by RT-qPCR and ddPCR targeting the N1 gene are found to be concordant (Figure S**3**; ZY, Pearson's correlation, $R = 0.98$, $P < 0.0001$; OG, Pearson's correlation, $R = 0.9$, $P < 0.0001$). Given the relative concordance between the RT-qPCR and ddPCR results, and the fact that that we have a richer data set across four target genes in duplicate reactions using RT-qPCR, we decided to carry out the rest of our analyses on the RT-qPCR results, alone; where relevant, associated analyses using ddPCR derived viral RNA concentrations are included in Data S**1** and are referenced below. We applied a logistic regression model that averaged RT-qPCR derived viral RNA concentrations over all four target genes and both sample collection kits with fixed effects to correct for systematic differences. The model uses a generalized estimating equations (GEE) approach and is described in the methods; it was used in all our primary analyses except where noted.

In study participants with uncomplicated COVID-19, the GEE model that considers RT-qPCR derived viral RNA concentrations across all four target genes in the gRNA shows that 49% [95% Confidence interval = 38 - 60%] of participants (n = 102) were positive for fecal SARS-CoV-2 RNA at the first time point around day 3 **(Fig. 2A)**. The proportion of participants with fecal shedding of SARS-CoV-2 RNA gradually declined to 40% (95% Confidence interval = 28 - 53%, n = 86) on day 14 and 11.0% (6 - 20%, 83) on day 28. To determine whether fecal SARS-CoV-2 RNA shedding continues after oropharyngeal shedding ceases, we then compared the presence of SARS-CoV-2 RNA in fecal samples to that in OP samples from the same participant[36]. At four months (120 days) post-enrollment, all participants (n = 57) who provided paired fecal and OP samples tested negative for SARS-CoV-2 RNA in their OP samples, but 12.7% [95% Confidence interval = 8.5% - 18.4%] of their fecal samples were positive for SARS-CoV-2 RNA **(Fig. 2B)**. OP samples were not tested beyond the four month time point. However, at seven months (210 days) post enrollment, 3.8% [2.0% - 7.3%] of the participants's fecal samples were positive for SARS-CoV-2 RNA. Among the 23 fecal samples collected at 10 months (300 days), none were positive for SARS-CoV-2 RNA. It should be noted that the presence of viral RNA in the feces at the later timepoints could be the consequence of prolonged infection and viral RNA shedding, or the consequence of a re-infection.

We then calculated the absolute concentrations of fecal SARS-CoV-2 RNA using RT-qPCR of samples collected in the ZY kit (**Fig. 2C;** corresponding data from samples collected in the OG kit are presented in Figure S**4)**. In samples collected around day 3, between 54 - 77 % of the participants shed viral RNA in their stool, depending on the gene targeted in the assay. At the first time point, looking at viral RNA concentrations derived from measuring the N1 gene, the

gene that yielded the most number of SARS-CoV-2 positive fecal samples at this time point, we find that positive stool samples have between 0.32 to 3.97 $\log_{10}$ copies of viral RNA per µL of eluate. We find that these viral RNA concentration data are concordant when measured using an orthogonal assay using ddPCR (Figure S**5)**. Finally, to understand the temporal dynamics of shedding, we treat time since enrollment in study as a continuous variable **(Fig. 2D)**, and observe a decline in fecal viral gRNA concentration over the first month post enrollment, with a few individuals demonstrating extended shedding vs. evidence of a possible re-infection at the four and seven month timepoints.

While gRNA is regularly used as an indicator of SARS-CoV-2 infection, this biomolecule does not mark an active infection, because non-infective viral particles can also harbor gRNA. Subgenomic RNA (sgRNA) is a possible indicator of an actively replicating virus, although there is ongoing debate about its specificity. Hence, we next quantified sgRNA[40]. 23.8% [95% Confidence interval = 15.2% - 35.3%] of participants had detectable sgRNA (0.8 to 5.69 $\log_{10}$ copies of viral sgRNA per µL of eluate) in the first time point after diagnosis (Figure S**6)**. This is in comparison to the 49.2% [38.2% - 60.3%] of participants who had detectable gRNA in the first time point after diagnosis. While there are samples that tested positive for gRNA that did not test positive for sgRNA, there were no samples where sgRNA was detected but gRNA was not. Finally, at the fourth time point, SARS-CoV-2 sgRNA had almost totally cleared with 0.7% [0.2% - 3.0%] of samples remaining positive for sgRNA.

Impact of interferon lambda on fecal shedding of SARS-CoV-2 RNA

As samples from this study were collected from individuals on a randomized controlled trial of Peginterferon-lambda, we carried out an exploratory analysis to determine whether this intervention affected fecal SARS-CoV-2 RNA clearance in the first month after treatment. We find that there is no significant difference in the percentage of participants who shed SARS-CoV-2 RNA in their feces between the two arms of the study at the first three time points **(Fig. 3A)**. We went on to calculate the odds ratio adjusted for age, sex, collection kit type and target gene (adjusted odds ratio, aOR) that a person who received the IFN-λ intervention would also be shedding viral RNA in stool at the first three time points **(Fig. 3B)**. At the first time point, around 3 days after enrollment in the study, we find that receiving the IFN-λ intervention is associated with lower odds of shedding viral RNA in stool (aOR = 0.32, 95% Confidence interval = 0.12 - 0.89). While the association between exposure to IFN-λ and lower odds of fecal viral RNA shedding is intriguing and suggests that exposure to the intervention on day 1 may decrease short-term fecal viral RNA shedding, this association failed to replicate upon execution of several sensitivity analyses (Figure S**7**; Data S**1**). In summary, in the current study we do not observe a robust effect of a single 180 mcg subcutaneous dose of IFN-λ on fecal SARS-CoV-2 RNA shedding.

Subjects with detectable fecal SARS-CoV-2 RNA also manifest gastrointestinal symptoms

In limited recent studies, the presence of fecal SARS-CoV-2 RNA has been linked to the presence of GI symptoms. However, these studies are mostly cross-sectional in nature, collect symptomatology data retrospectively and do not use a uniform, benchmarked methodology for

quantification of SARS-CoV-2 RNA in stool. To address the question of whether fecal viral RNA shedding is associated with GI symptoms, we collected comprehensive longitudinal symptomatology data, including information on GI symptoms, from study participants in this interventional trial and compared these to absolute viral RNA concentrations measured in their feces (**Fig. 4A)**. Across the first month of the study, we find that participants who shed viral RNA in their stool were more likely to report nausea (aOR = 1.61, 95% Confidence interval = 1.09 - 2.39), vomiting (3.20, 1.11 - 9.21) and abdominal pain (2.05, 1.09 - 3.86); no association was observed between viral RNA shedding and diarrhea (1.10, 0.63 - 1.91), or when considering any GI symptom (1.38, 0.94 - 2.04). Respiratory and systemic symptoms including runny nose (1.67, 1.05 - 2.66), headaches (1.56, 1.04 - 2.35) and body aches (2.21, 1.45 - 3.38) are also associated with the presence of fecal SARS-CoV-2 RNA. Taken together, fecal SARS-CoV-2 RNA shedding is positively associated with most GI symptoms and with specific systemic and respiratory symptoms.

To determine whether the observed association between symptoms and fecal shedding was independent of respiratory shedding, we next divided the data into two subsets, based on whether or not the participant was shedding virus in the oropharynx at the time the fecal sample was taken; specifically, we looked at participants whose OP swabs collected within 3 days of the stool sample (a) did not have any detectable SARS-CoV-2 RNA (n = 69; **Fig. 4B),** or (b) had detectable SARS-CoV-2 RNA (n = 54; **Fig. 4C**). Participants who were shedding viral RNA from the oropharynx had higher rates of almost all of COVID-19-related symptoms, and we found no significant association between fecal shedding and symptoms for this subgroup. By contrast, participants who were not shedding viral RNA from the oropharynx had far lower rates

of COVID-19-related symptoms in general, but we found many significant associations between fecal shedding and symptoms for this subgroup. This is consistent with an interpretation where patients with an active infection of the respiratory system experience an array of COVID-19-related symptoms independent of whether or not they are fecal shedding, but where patients whose respiratory infection has cleared could still be experiencing an active infection of the GI tract, which itself is associated many different COVID-19-related symptoms. Taken together, these data suggest that fecal shedding of SARS-CoV-2 RNA is a possible indicator of an ongoing GI infection, and that this infection is accompanied by GI and other systemic symptoms.

Discussion

Severe SARS-CoV-2 infections can lead to a life-threatening hypoxemic respiratory failure. Therefore, much of the initial investigation of COVID-19 focused on the respiratory infection and related manifestations of the disease. This may be why, two years into the pandemic, we still do not definitively know whether SARS-CoV-2 infects the GI tract of humans. However, we know that SARS-CoV-2 can infect intestinal cells in vitro - both in cell lines[33] and human tissue-derived intestinal organoids.[30–32] Additionally, the largest autopsy series of patients with COVID-19, to date, recently demonstrated consistent evidence of infection of the small intestine by SARS-CoV-2; they also recovered live virus from these intestinal biopsies.[5] This evidence suggests that SARS-CoV-2 can infect the GI tract, and perhaps when it does, it induces the GI symptoms observed in individuals with COVID-19. This postulated GI-tropism of SARS-CoV-2 is in keeping with the fact that other *Betacoronaviruses* that infect mammals can cause GI diseases. For example, BCoV, causes severe GI diseases such as calf diarrhea and winter dysentery in cows.[2,41] What we have lacked in trying to understand whether the GI tract is commonly infected in COVID-19 is longitudinal samples that demonstrate prolonged shedding of fecal viral RNA after respiratory shedding has stopped. We have also lacked the data that would enable us to clearly investigate whether or not there is a link between fecal viral RNA shedding and GI symptoms, both during and after respiratory infection by SARS-CoV-2.

To address this gap, we leveraged one of the largest collections of longitudinal fecal samples from patients with mild to moderate COVID-19 to investigate fecal viral RNA shedding and its relationship to both OP viral RNA shedding and COVID-19 symptoms. Among 113

participants who provided stool samples in this study, 49.2% [95% Confidence interval = 38.2% - 60.3%] shed viral RNA in their feces within 6 days after their COVID-19 diagnosis. The fact that only a subset of individuals with COVID-19 exhibited fecal viral RNA shedding may be the consequence of a broad, nearly one week, window for the first sample collection from the time of diagnosis; alternatively, this may also be the result of physiological and genetic differences between individuals. Over the course of the first month in this study, the number of participants shedding fecal viral RNA decreased to 11% [6 - 20%], and the viral RNA concentration among those still shedding decreased from up to ~3 $\log_{10}$ copies per µL to < 1 $\log_{10}$ copies per µL. At the first time point, we find that a larger proportion of participants shed viral RNA in their OP swab compared to their feces; however, this trend reverses in the rest of the time points. This suggests that clearance of SARS-CoV-2 is more rapid in the respiratory tissue than it is in the GI tissue and that the GI tract may be a site of longer term infection.

When considered in the context of previously documented evidence of a likely GI infection by SARS-CoV-2, our detection of SARS-CoV-2 sgRNA in fecal samples supports the model of an active infection in the GI tract. The presence of sgRNA, as opposed to gRNA, has been proposed as a marker of active infection and viral replication; however, subsequent work has now established that sgRNA outlives actively replicating virus in cell culture experiments, and therefore may be an unreliable indicator of an ongoing, active infection.[40,42] Therefore, while we detect sgRNA in stool up to 28 days after infection, whether or not this, on its own, is sufficient evidence of an ongoing infection remains unclear.

Beyond informing our understanding of SARS-CoV-2 pathobiology, the information we present on the frequency, amount and duration of viral RNA shed in stool is valuable for inferring population-level prevalence of COVID-19 from wastewater studies. This may in turn help inform public health measures. For example, long-term fecal viral RNA shedders may contribute to prolonged elevated levels of SARS-CoV-2 RNA in wastewater. If transmission occurs largely or entirely through respiratory secretions, the continued presence of fecal viral RNA in wastewater from a prolonged GI infection, may be mistakenly interpreted as evidence of the prevalence of infectious individuals in a community. Since wastewater viral RNA levels are being considered for use in guiding community level policies (e.g. shutdowns and reopenings),[43–47] it is critical that we understand how respiratory viral shedding and transmissibility of SARS-CoV-2 RNA are temporally related to fecal viral RNA shedding.

Based on the available evidence, it is highly plausible that the presence of GI symptoms in patients with COVID-19 is due to infection of the GI tissues. With a comprehensive collection of clinical symptom data and fecal viral RNA concentrations, we find that over the course of the first month after enrollment, those who shed viral RNA in stool are more likely to also have GI symptoms including nausea, vomiting and abdominal pain among other symptoms like runny nose, body aches and headaches. It is notable that those who shed viral RNA in stool were not more likely to have diarrhea - this finding is contradicted by two prior studies (n = 59, 44) that found that patients with diarrhea were more likely to shed viral RNA in stool and, that too, at higher concentrations.[3] Our finding of no association between diarrhea and fecal viral RNA shedding might be due to the relatively small number of participants who reported diarrhea in our study. When focusing on participants who had extended shedding of viral RNA in their stool

even after their OP shedding had ceased, we found that fecal shedding of viral RNA is associated with a range of systemic and GI symptoms. On the other hand, for the duration that participants provided an OP swab positive for viral RNA i.e. had an active respiratory infection, we do not find any association between fecal viral RNA shedding and symptomatology. We postulate that this is because participants who have an ongoing respiratory infection manifest classic COVID-19 related symptoms, whether or not they have an infection in their GI tract. These observations support the hypothesis that there is likely a prolonged SARS-CoV-2 infection of the GI tract even after the upper respiratory infection is cleared. Since the GI tract is a highly immunoactive tissue,[48] prolonged infections of the GI tissue may have consequences to patient health and also be associated with the hitherto mysterious phenomenon of PASC or 'Long COVID'. In fact, many studies following patients who have recovered from COVID-19 identify the prolonged presence of a gastrointestinal sequelae.[49–55]

In conclusion, we sought to address a key gap in our knowledge about the pathophysiology of a possible GI infection by SARS-CoV-2 by sampling stool over an extended period of time (10 months) and gathering paired symptomatology data. We have demonstrated the longest recorded shedding of fecal SARS-CoV-2 RNA in any COVID-19 patient out ~210 days post-infection in two participants. Further, we have found that extended shedding of SARS-CoV-2 RNA in participants who no longer have detectable viral RNA in OP swabs is closely associated with a host of systemic and GI symptoms, providing further evidence of a SARS-CoV-2 infection of the gut. Data presented here, when placed in the context of preliminary work that has suggested that the extended presence of SARS-CoV-2 viral antigen in gut biopsies from participants with COVID-19 may be associated with an improved immune response,[22] urges

follow up immunological studies that investigate stool samples. Finally, initiatives such as RECOVER that are poised to elucidate the hitherto elusive phenomenon of PASC should look closely at stool samples as an important factor of SARS-CoV-2 infection with potential long term impact.

Limitations of Study

Despite its large size and longitudinal nature, this study has limitations. First, the study is limited in its resolution, having only collected six samples over a 10-month period. Follow up studies with more frequent sampling, especially in the first two months after diagnosis, may allow a more nuanced model of decline of fecal viral RNA concentration. This will also allow a closer evaluation of the relative cessation of viral RNA in stool *vis-a-vis* other respiratory samples such as the OP swab. We were also unable to collect stool samples in a way that would enable recovery of live virus. As this was an outpatient study during the early part of the pandemic, we required participants to collect stool themselves at home, and then mail the stool kits to us. For safety and practical purposes, we thus had to provide participants with kits that were rated for virus inactivation. Future studies, which facilitate the careful, consistent collection of stool samples from individuals with COVID-19 in a safe setting, might enhance the likelihood of more accurate measurement of live virus. This would be more direct evidence of SARS-CoV-2 being viable in the gut. Third, we did not obtain direct tissue evidence of infection - to do so would require intestinal biopsies. Of note, recent autopsy[5] and prior biopsy-based[22] reports in limited numbers of patients have demonstrated evidence of direct intestinal infection and cytopathic changes. While intestinal biopsies from patients with mild to moderate COVID-19 would be highly informative, to date these samples have been understandably difficult to obtain. In upcoming large studies, such as the Researching COVID to Enhance Recovery (RECOVER, NIH) study, a subset of patients will be getting such biopsies, and the results of these large-scale studies will be illuminating.

Finally, it would be interesting to sequence fecal viral RNA from participants with extended shedding to evaluate for persistence of the original virus variant, evolution of the

original variant, and/or a potential re-infection by the same or a different SARS-CoV-2 variant. Unfortunately, one of the limitations of current technologies for sequencing variants from complex matrices such as stool is the requirement of an adequate concentration of virus to be able to either amplify or assemble the virus from direct or enriched sequencing. As future technologies are developed for sensitive determination of variant sequences from stool, this type of analysis should be feasible. Of note, this study was carried out prior to the emergence of the strains (omicron, delta) that are prevalent today. Different strains may have different relative tropisms to the respiratory versus GI tract and may exhibit differences in clearance rates. This may be the consequence of their inherent biology, as well as the immune status of the host due to underlying disorders, prior COVID-19 disease and natural immunization, or vaccination.

Of note, in this study we were limited to samples that were collected as part of a previously published clinical trial [36]. The original study reports the enrollment criteria applied to recruit participants. Briefly, the study actively sought to have equal male and female, racially and socio-economically diverse participants between the ages of 18 to 75. The study did not collect information about self-reported gender in recruitment. Participants at risk of current or imminent hospitalization, with respiratory rate >20 breaths per minute, room air oxygen saturation <94%, history of decompensated liver disease, recent use of interferons, antibiotics, anticoagulants or other investigational and/or immunomodulatory agents for treatment of COVID-19, and prespecified lab abnormalities were excluded. Additionally, pregnant or breastfeeding participants were also excluded.

STAR Methods

Key resources table

Resource availability

Lead contact: Supplementary Information is available for this manuscript. Correspondence and requests for materials should be addressed to the lead contact, Ami S. Bhatt (269 Campus Dr, CCSR 1155b, Stanford University, Palo Alto, CA 94305. Tel: (650) 498-4438; Email: asbhatt@stanford.edu).

Materials availability: PCR primers sequences are reported in Data S**1**. Other resources are available upon request of the lead contact.

Data and code availability:

- All data have been deposited at Zenodo (https://zenodo.org/record/6374138) and are publicly available as of the date of publication.
- All custom code and mathematical models have been deposited at Zenodo (https://zenodo.org/record/6374138) and are publicly available as of the date of publication.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

Experimental Model and Subject Details

Study design and population: A total of 120 adults aged 18 - 71 years who had received a positive SARS-CoV-2 reverse-transcriptase quantitative polymerase chain reaction (RT-qPCR) based respiratory swab test nasal test within the past 72 hours were recruited for enrollment in Lambda (NCT04331899), a single-blind, placebo controlled, phase 2 clinical trial of Peginterferon Lambda-1a (IFN-λ) as an intervention for uncomplicated coronavirus disease 2019 (COVID-19). Informed consent was obtained for all participants under Stanford University Institutional Review Board (IRB) approved protocol # 55619 (PIs: Upinder Singh, Prasanna Jagannathan).

The primary results of the null study, secondary outcomes, and the full details of study recruitment, inclusion and exclusion criteria were previously reported on and are only briefly summarized here.[36] Individuals with study defined lab abnormalities, respiratory rate >20 breaths per minute, room air oxygen saturation levels <94%, pregnancy or breastfeeding, or recent history of hospitalization, uncontrolled liver disease, or use of COVID-19 interventional therapeutics, anticoagulants, antibiotics, and/or antivirals were excluded from the study. Subjects were randomized 1:1 to either the interventional or control study arm to receive a one-time subcutaneous injection of Peginterferon Lambda-1a or saline, respectively, on the first day of enrollment. Randomization was stratified by age (≥ 50 and < 50 years old) and sex. The demographics of study participants are summarized in Table 1. Participant information on sex, age, race and ethnicity was self-reported and was reported in the original clinical manuscript describing this study.[36] Information on gender and socioeconomic status was not collected.

In addition, healthy adults were recruited to provide stool samples for use as extraction controls under Stanford IRB protocol #42043 (PI: Ami Bhatt). All donors gave informed consent prior to donating stool samples. Information on sex, gender, age, socioeconomic status, race and ethnicity was not collected.

Trial Registration: ClinicalTrials.gov Identifier: NCT04331899

Methods Details

Study samples and data: Stool and other data and samples were collected from each set of study participants as outlined below.

For the first 28 days following enrollment, participants in the clinical trial completed daily symptom questionnaires administered via REDCap Cloud (version 1.5)[36] and self-performed daily measurements of temperature and oxygen saturation using study provided at-home devices. Participants returned to the study site on 1, 3, 5, 7, 10, 14, 21, 28 days (all +/- 1 day) and 120, 210, and 300 days (all +/- 3 weeks) post-enrollment for follow-up visits during which oropharyngeal (OP) swabs were collected, symptoms were queried, and vital signs were recorded. All clinical trial participants were provided a fecal sample collection kit on 0, 5, 21, 28, 120, and 210 days after enrollment and were asked to collect a stool sample in the provided kit, store at room temperature, and drop off for processing at their subsequent study visit or mail back to the study site at the long term follow up time points. We define the following six time points based on when participants returned the stool samples: days 3 (range 0 - 7 days), 14 (8 - 21), 28 (22 - 35), 120 (75 - 165), 210 (166 - 255) and 300 (>255) (**Fig. 1A**).

At the start of study enrollment on 25 April 2020, the collection kit consisted of the OMNIGene GUT collection tube (OG), toilet accessory, gloves and Spanish and English translations of manufacturer instructions. Later, starting 14 May 2020, the Zymo DNA/RNA shield fecal collection tube (ZY) was included in the fecal sample collection kit in addition to the OG collection tube. Spanish and English translations of manufacturer instructions specific to the ZY collection tube were also added. Subsequently, all participants were asked to collect a portion of the same stool sample in both of the two kits for each time point.

The OG and ZY collection tubes are both marketed to preserve stool samples at ambient temperatures for up to 30 days. This eliminated the burden of sample refrigeration requirements for study participants. Fecal samples were processed within 24 hours of receipt by the lab. Samples collected in the OG and ZY collection tubes were processed similarly, by first vortexing the collection tube for 30 seconds to thoroughly homogenize the sample. Each sample was then aliquoted into 1.8 mL cryovials, labeled with the patient study ID and study time point, and then frozen at -80 °C.

Healthy control stool samples for use in every batch of RNA extractions were obtained from a healthy individual without prior history of COVID-19 exposure or positive SARS-CoV-2 respiratory test. Healthy stool samples for the limit of blank (LoB) determination were collected in 2018 well prior to the onset of the pandemic. All healthy donors self-collected fecal samples fresh and stored them at 4 °C until processing. Within 24 hours of sample collection samples were aliquoted into cryovials without preservative and frozen immediately at -80 °C.

535 Extraction of RNA: Stool samples were randomly assigned a sample ID and processed for RNA
536 extraction in batches of 18 following a previously optimized method,[11,38] which is summarized
537 here and in Figure S**1**.
538
539 Two positive controls (OG and ZY) were included in each extraction batch for a total of
540 20 extractions per batch. Positive controls were prepared by adding biopsy punches of stool
541 collected from a healthy individual to OG (4 biopsy punches) and ZY (8 biopsy punches) tubes.
542 Each tube was then spiked with 10 µL of synthetic SARS-CoV-2 RNA at 10^4 copies/ µL,
543 vortexed for 30 seconds for homogenization, transferred in 500 µL aliquots to eppendorf tubes
544 and frozen -80 °C.
545
546 Samples were gradually thawed on ice and vortexed for five seconds to ensure thorough
547 homogenization. 500 µL of the stool-buffer slurry was transferred to an eppendorf tube, spun at
548 10,000 x g for 2 minutes at room temperature, and 140 µL of the supernatant was transferred to a
549 fresh eppendorf tube for RNA extraction using the QiaAMP Viral RNA Mini kit. RNA
550 extraction was performed as per manufacturer's protocol and eluted in 100 µL of the elution
551 buffer EB from the kit. Extracted RNA was then transferred to 96 well plates, briefly spun down,
552 sealed and stored at -80°C until further analysis.
553
554 Samples collected at the 4, 7 and 10 month timepoints and associated batch controls were
555 additionally spiked with 10 µL of attenuated BCoV vaccine as recommended.[11] BCoV was
556 prepared by resuspending one vial of lyophilized Zoetis Calf-Guard Bovine Rotavirus-
557 Coronavirus Vaccine in 3 mL of phosphate buffered saline as per the manufacturer's instructions.

558

559 **Quantification and Statistical Analysis**

560 RT-qPCR quantification of RNA: An RT-qPCR assay to detect and quantify SARS-CoV-2
561 genomic RNA (gRNA) was developed using primer probe sets recommended by the United
562 States Centers for Disease Control and prevention (CDC)[56] targeting the Envelope protein (E),
563 Nucleocapsid proteins (N1, N2), and RNA-dependent RNA polymerase protein (RdRP) of the
564 viral genome. To quantify SARS-CoV-2 subgenomic RNA (sgRNA) from stool samples as
565 previously described[40] an additional primer probe set targeting the N1 gene with the forward
566 primer annealing to the canonical leader sequence at the 5' end was included in the assay. All
567 RNA extracts were assayed for all four gRNA targets and the single sgRNA target. Primer and
568 probe sequences are listed in Data S**1**.

569

570 Each 20 µL RT-qPCR reaction was composed of 5 µL TaqPath 1-Step RT-qPCR Master
571 Mix, CG, 1.5 µL of primer/probe mix, 8.5 µL of nuclease-free water. The primer/probe mix was
572 prepared with a final concentration of 400 nM of each of the forward and reverse primers and
573 200 nM of the corresponding probe in 8.5 mM Tris-HCl pH 8.0 and 0.8 mM EDTA. Reactions
574 were prepared in MicroAmp Optical 384-well plates with 5 µL of stool RNA samples, synthetic
575 RNA standards, or nuclease free water using a Biomek-FX liquid handler. Every assay plate also
576 included standard curves. Standard curves were prepared by serially diluting quantitative
577 synthetic SARS-CoV-2 RNA from 10^{5}-10^{-1} copies per µL. For standard curves in the sgRNA
578 assays, a purified PCR product corresponding to the target gene[40] was diluted from 10^{6}-10^{-1}
579 copies per reaction. Nuclease-free water was used as a negative control.

580

RNA extracted from each stool sample was assayed in two technical replicates for each target. Standard curves were run in technical duplicates for all targets on every RT-qPCR assay plate. Eight negative controls were included in each assay plate. Prior to the assay, plates were sealed with an optically clear seal and spun down at room temperature. The samples were assayed in a 12k Flex Applied Biosystems qPCR machine in standard mode using the following cycling conditions: 25 °C for 2 minutes, 50 °C for 15 minutes, and 95 °C for 2 minutes, followed by 45 cycles of 95 °C, 3 seconds, and 55 °C, 30 seconds.

In the RT-qPCR assays, quantification cycle (Cq) value was calculated using the Design and Analysis software. On a plate-by-plate basis, assays with a Cq value greater than the Cq value of the synthetic RNA standard at 1 copy per µL were called undetermined. Cq values for each sample were converted to viral RNA concentration in copies/µL using the linear regression model fit to the standard curve for each plate. We used a statistical model to average over the results of all the technical replicates, and more details about the model are available in the Statistical analysis section.

Finally, we calculated the LoB of the assay (more details available in the STAR methods) and converted all viral RNA concentrations equal to or lower than the LoB to be undetermined, because these were beyond the reliable specificity of the assay. All viral RNA concentrations were expressed on a logarithmic scale by applying the transformation $\log_{10}$(viral RNA concentration+1).

603 SARS-CoV-2 viral RNA concentrations from oropharyngeal swabs were derived from a
604 previously published companion study.[36] This study measured the E gene in the SARS-CoV-2
605 genomic RNA and RNaseP in the human genome in a multiplexed assay. RNaseP was used as an
606 internal control for the extraction of RNA and to monitor the effect of RT-qPCR inhibitors in
607 these samples. Only samples where RNaseP was detected were evaluated. As a requirement for
608 the Stanford FDA Emergency Use Authorization for the SARS-CoV-2 RNA diagnostic test, the
609 sensitivity of the assay for nasopharyngeal swab testing was determined to be 1000 copies/mL.
610 While the FDA did not require the assessment of assay sensitivity for different respiratory
611 tissues, we believe that the assay sensitivity for nasopharyngeal vs. oropharyngeal swabs to be
612 comparable. Similarly, based on previously reported benchmarking and Limit of Detection
613 (LoD) assays, the sensitivity of fecal sample testing for SARS-CoV-2 RNA is 1000
614 copies/mL.[11] Moreover, the assay sensitivity of fecal testing was highly concordant between the
615 tested genes, particularly for the N1, N2, and E genes; RdRP has a slightly lower sensitivity by
616 comparison.[11] Therefore, we are confident that the sensitivity of SARS-CoV-2 RNA testing is
617 highly comparable in stool and respiratory biospecimen of the study subjects (1000 copies/mL).
618
619 ddPCR quantification of RNA: Droplet digital PCR (ddPCR) is resilient to PCR inhibitors
620 prevalent in stool, enables absolute quantification without the need for an exhaustive standard
621 curve, and is also more sensitive than traditional qPCR.[11,38] Therefore, we quantified viral RNA
622 using this orthogonal method as previously described.[11] The ddPCR reactions were prepared
623 with the One-Step RT-ddPCR Advanced Kit for Probes. Using a Biomek FX liquid handler, each
624 reaction well was loaded with 5.5 µL of extracted RNA to 5.5 µL Supermix, 2.2 µL reverse
625 transcriptase, 1.1 µL of 300 nM dithiothreitol (DTT), 1.1 µL of 20× Custom ddPCR Assay

626 Primer/Probe Mix and 6.6 µL of nuclease-free water per the manufacturer instructions. For
627 multiplexed reactions, we added 1.1 µL of each of the primer/probe mixes and reduced the
628 amount of nuclease free water to 5.5 µL.
629
630 We then used a QX200 AutoDG Droplet Digital PCR System to partition reaction
631 samples into droplets of 1 nL using default settings. PCR amplification of the templates was
632 performed on a BioRad T100 thermocycler using the following thermocycling program: 50 °C
633 for 60 min, 95 °C for 10 min, 40 cycles of 94 °C for 30 s and 55 °C for 1 min, followed by 1
634 cycle of 98 °C for 10 min and 4 °C for 30 min with ramp speed of 1.6 °C/s at each step. Finally,
635 amplified reactions were quantified using a ddPCR reader.
636
637 The ddPCR analysis was guided by the Droplet Digital PCR Applications Guide on
638 QX200 machines (BioRad)[57] and the digital MIQE guidelines.[58] We have included the
639 recommended associated checklist in Data S**1**. We applied a rigorous strategy to threshold the
640 assays and identify true positive reactions as previously described[11] and summarized below.
641 Briefly, we analyzed the standards and negative controls in a plate-by-plate fashion and applied a
642 suitable threshold to these samples. This threshold was applied such that the number of positive
643 droplets in the negative control was minimal and the concentration of RNA in the standard
644 matched the theoretical expectation most closely. We then calculated the difference in amplitude
645 between the negative droplets and the threshold in the reactions with the negative control, and
646 applied a threshold to all the other wells such that this same difference in amplitude was
647 maintained. Finally, as with the RT-qPCR reactions, we established an LoB for this assay (more
648 details available in STAR methods), and any sample with viral RNA concentration less than or

equal to the LoB was considered to be undetermined. All viral RNA concentrations were expressed on a logarithmic scale by applying the transformation $\log_{10}$(viral RNA concentration+1).

Ensuring high specificity in RT-qPCR and ddPCR assays of fecal SARS-CoV-2 RNA: In assays to quantify viral RNA, we took a conservative approach at every step to ensure high specificity. First, we adopted a method to determine the limit of blank (LoB) that is based on guidelines set out by the Clinical and Laboratory Standards Institute (CLSI)[63], as summarized in the next section. We systematically identified the LoB for stool collected in the OG and ZY kits against each of the four target genes in independent combinations. All samples with an RNA concentration equal to or lower than the corresponding LoB are considered to have an undetermined amount of viral RNA, since this is below a reliable specificity threshold for that assay (example in Data S**1)**. Second, we identified the linear detection range of our assays. A six-point 10-fold dilution series of synthetic SARS-CoV-2 RNA from the American Type Culture Collection (ATCC) starting at 10^4 $\log_{10}$ copies per µL was used here as previously described[11]. Resulting standard curves generated for each of the genes in the genomic RNA measured using RT-qPCR and those measured by ddPCR are shown in Data S**1**. In assays that detected sgRNA, we used a six-point 10-fold dilution series with pre-quantified sgRNA starting at 10^6 $\log_{10}$ copies per µL from a previously reported study[40] and provide standard curves in Data S**1.** All samples that yield a viral RNA concentration below the lowest detectable concentration in the linear range of standards are considered to have an undetermined amount of viral RNA. Third, anticipating that few if any stool samples collected beyond the 28 day time point were going to be positive for SARS-CoV-2 RNA, we incorporated a control to guard against false negatives

672 that could result from incomplete or inefficient extraction of RNA, as previously described[11].
673 Briefly, all long-term stool samples were spiked with 10 µl of attenuated Bovine coronavirus
674 (BCoV) prior to RNA extraction. The extracted RNA was then tested for the M gene from BCoV
675 in addition to the regular SARS-CoV-2 based assays. This served to determine if RNA
676 extractions were successful, ensuring we did not falsely report negative SARS-CoV-2 assays as a
677 consequence of ineffective RNA extraction. Out of 239 samples, 237 yielded BCoV RNA, and
678 those that did not were left out of further analysis. Together, these experimental checkpoints
679 increase confident that our reported fecal viral RNA concentrations are accurate.
680
681 Estimating limits of blanks: Understanding the specificity of the assays used in this study to
682 quantify viral RNA is critical to evaluate confidence in results derived thereof. Therefore, we
683 used a strategy based on guidelines set out by the Clinical and Laboratory Standards Institute
684 (CLSI)[63] to quantify the limit of blank (LoB) of our stool preservation and detection protocol.
685
686 To this end, we used stool samples collected from four healthy donors in the Fall of 2018. Since
687 this was from before the emergence of SARS-CoV-2, these samples are confidently negative for
688 SARS-CoV-2 RNA. One stool sample from each of the four donors was aliquoted into separate
689 OG and ZY tubes as per manufacturer instructions. This was performed in independent
690 duplicates by two different operators yielding 16 stool samples. Next, RNA was extracted from
691 each of these samples in duplicate by the two operators resulting in 64 total RNA extracts. The
692 sample preparation protocol is summarized in Data S**1**.
693

694 The 64 RNA extracts were assayed for the E, N1, N2 and RdRP genes in the gRNA in duplicate
695 reactions identical to how clinical samples were assayed in this study. Next, these samples were
696 also assayed for the N1 genes in ddPCR assays. Taken together, we calculated the LoB for
697 relevant combinations of stool preservation (OG, ZY), target gene (E, N1, N2 and RdRP), and
698 detection method (RT-qPCR, ddPCR).
699
700 It was notable that across all targeted genes in both RT-qPCR and ddPCR assays, the LoB
701 measured in the OG kit was higher than that measured in the ZY kit. Specifically, RT-qPCR
702 assays targeting the N1 gene yielded 0.487 $\log_{10}$ copies per µL of viral RNA in samples
703 preserved in OG and 0.237 $\log_{10}$ copies per µL of RNA in those preserved in ZY. These
704 corresponded to 0.429 copies per µL and 0.164 copies per µL of RNA in ddPCR assays targeting
705 the N1 gene. Finally, while targeting the N2 gene via RT-qPCR also yielded low RNA
706 concentrations in these negative controls, E and RdRP were highly specific and yielded no
707 detectable RNA for these targets in the negative controls (Data S**1**). The RNA concentration
708 derived here is used as the LoB in all further data analysis. Thus, all samples that bear an RNA
709 concentration equal to or lower than the corresponding LoB are considered to have an
710 undetermined amount of viral RNA, since this is below a reliable specificity threshold for that
711 assay (example in Data S**1**).
712
713 Guarding against PCR inhibitors for the reliable detection of viral RNA: Aware of the presence
714 of potential PCR inhibitors in the stool matrix, we wanted to estimate the degree to which our
715 RT-qPCR assays were impacted by PCR inhibition. We posited that diluting the stool RNA
716 extracts prior to assaying for SARS-CoV-2 RNA would dilute any potential PCR inhibitors

717 derived from the stool matrix. Thus, we would expect a higher positivity rate from assaying the
718 diluted extracts. To this end, we assayed 72 clinical samples by RT-qPCR at the concentration
719 they were extracted at (1X), and at a ten-fold dilution of the same samples (0.1X). In aggregate
720 across the 4 RT-qPCR target genes, assaying the samples at 0.1X resulted in a gain of 4 positive
721 samples but a loss of 15 positive samples, likely due to viral RNA concentration falling below
722 the detection limit of the RT-qPCR assay with dilution (Data S**1**). Thus, the RT-qPCR analysis
723 of the stool RNA extracts likely does not exhibit a high degree of PCR inhibition.
724
725 Statistical analysis: Absolute standardized differences (ASD),[59] expressed in units of standard
726 deviations, are displayed in **Table 1** to compare the distribution of characteristics in participants
727 reporting GI symptoms at enrollment or not. We interpreted ASDs using Cohen's guidelines (d:
728 0.2 = small difference; 0.5 = medium difference; 0.8 = large difference; d < 0.2 = trivial
729 difference).[60]
730
731 Our primary statistical analyses examined associations between participant characteristics
732 and whether the RT-qPCR based detection of SARS-CoV-2 gRNA was positive, focusing only
733 on the stool samples collected during the main study at the first three time points, and including
734 fixed effects to account for the different positivity rates of the four target genes (E, N1, N2 and
735 RdRP) and the two collection kits (OG and ZY). We augmented this with two sensitivity
736 analyses. First, we conducted a subgroup analysis that included samples from all six time-points
737 but that focused on the subset of participants who returned at least one sample during the long-
738 term follow-up; we made decision to focus our primary analysis on the first three time points and
739 to supplement it with this sensitivity analysis to avoid the concern that the decision to join the

740 extended study might correlate with certain patient risk. Second, we conducted subset analyses
741 that focused on individual genes separately. In all cases, we used logistic regression models fit
742 with generalized estimating equations (GEE)[61] to account for the correlation between samples
743 and replicates within a participant.
744
745 To examine whether Peginterferon Lambda-1a (IFN-λ) had an effect on fecal viral RNA
746 shedding, we fit a logistic regression to estimate the odds ratio of fecal shedding in participants
747 receiving the IFN-λ intervention versus those that received a saline placebo. We adjusted the
748 odds ratio by collection kit type (OG and ZY) and gene (E, N1, N2 and RdRP), to account for
749 systematic differences between measurements, and as well as by the patient's age and sex,
750 because randomization had been stratified by those features.[62] We included statistical interaction
751 terms between study arms and indicators for time of collection in the model to estimate the
752 difference between study arms at each time of collection. In addition to the two sensitivity
753 analyses described above, we also used a negative binomial model to assess the association
754 between the IFN-λ intervention and the total viral RNA concentration, whereas before we used
755 GEE to account for correlation within individual patients.
756
757 In analyses to estimate association between fecal SARS-CoV-2 RNA and symptoms, we
758 regressed the presence of symptoms reported at the time of sample collection on an indicator of
759 the presence of fecal SARS-CoV-2 RNA, adjusted for age, sex, log of the number of days since
760 symptom onset, collection kit type (OG and ZY), and gene (E, N1, N2 and RdRP). We fit a
761 separate logistic regression for each of the symptoms. We additionally fit models including an
762 interaction between fecal SARS-CoV-2 RNA shedding and an indicator of OP shedding to

763 estimate associations among participants with or without an ongoing presence of viral RNA in
764 their OP swabs.
765
766 All tests were two-sided and conducted at the 0.05 level of significance. Analyses were
767 performed in Python version 3.8.5, using the Statsmodel package, version 0.12.0.
768
769 IFN-λ does not impact fecal SARS-CoV-2 RNA shedding: Exposure to IFN-λ appears to present
770 lower odds of fecal viral RNA shedding at the first time point, around 3 days after receiving the
771 intervention **(Fig. 3B)**. However, this association failed to replicate upon closer examination
772 using several sensitivity analyses, as follows.
773
774 1) We calculated the adjusted odds ratio (aOR) that a person who received the IFN-λ intervention
775 would also be shedding viral RNA in stool at the first three time points, limiting our attention to
776 the subset of individuals who elected to participate in the extended study. Amongst these
777 participants there was no association between the intervention and fecal shedding during any of
778 the six time points (**Figure S7A, B**).
779
780 2) We looked at an analysis that was restricted to just individual genes and kits. In this analysis,
781 we find that the association at the first time point is being driven entirely by samples collected in
782 the OG kit, which has previously been shown to have lower sensitivity for fecal SARS-CoV-2
783 RNA detection[11] (Data S**1**).
784

785 3) An analysis that looked at viral RNA concentrations instead of binary test results (positive vs.
786 negative) found no association at any of the three time points (**Figure S7C**).
787

Acknowledgements

We thank Alexandria Boehm, Marlene Wolfe and Nasa Sinnott-Armstrong for guidance on processing stool samples and detection of RNA; Angela Rogers for providing stool samples from participants admitted at Stanford Hospital; Rebecca Osbourne, Tiffany Nguyen and the members of the Stanford Clinical and Translational Research Unit for assistance with stool sample receipt and processing; Elizabeth Ponder for coordinating initial stool sample collection kit distribution to study participants and providing information about funding from Chem-H; Catherine Blish and members of the Blish Lab for receiving and temporary storage of stool samples prior to biobanking; Dean Felsher for access to the QuantStudio 12K Flex qPCR machine; Yvonne Maldonado and Jonathan Altamirano for helping acquire funding to support toward this work; Said Attiya and Dhananjay Wagh for guidance on applying ddPCR assays; David Solow-Cordero for assistance setting up the Biomek FX and providing access; and Luisa Jiminez and Sopheak Sim for assistance in using the Stanford Functional Genomics Facility and High-Throughput Bioscience Center and Frida Salcedo for help acquiring reagents from BioRad. We are grateful to the Peginterferon-λ1a clinical trial team for coordinating procurement of stool samples from outpatients enrolled in this trial. Biorender has been a valuable resource for creating schematic illustrations. This work was supported by a ChemH-IMA grant (to A.S.B. and P.J.), the Stanford Dean's Postdoctoral Fellowship (to A.N.), an AACR Fellowship (to S.Z.), and an NSF Graduate research fellowship program grant (to A.H. and D.T.S.). The laboratory of A.S.B. is supported by NIH R01 AI148623 and R01 AI143757, and H.H is supported by an NIH award UL1TR003142.

Author contributions and data oversight

A.N., S.Z., E.F.B. and S.E.V. contributed equally to this work. A.N. designed experiments, extracted RNA from stool samples, assayed viral RNA using RT-qPCR and ddPCR, analyzed data and wrote the manuscript. S.Z. designed experiments, assayed viral RNA using RT-qPCR and ddPCR, analyzed data, generated plots in R and wrote the manuscript. E.F.B. and S.E.V. designed experiments, biobanked stool samples, extracted RNA from stool samples and wrote the manuscript. A.D. and H.H. designed experiments, performed statistical analyses, generated plots in Python and wrote the manuscript. R.M.P. analyzed data. A.H., D.T.S. and R.V. helped design experiments. K.B.J., J.P., H.F.B., U.S., B.A.P., J.A. and P.J. helped collect samples through the Lambda clinical trial and guided data analysis. A.S.B. helped design experiments, analyze data, and wrote the manuscript. S.Z., A.D. and H.H. performed and replicated the statistical analysis. A.N., E.F.B., S.E.V, and A.S.B. oversaw the statistical analysis. A.N., S.Z., E.F.B., S.E.V., A.D., H.H., and A.S.B. have unrestricted access to all data. A.N., S.Z., E.F.B., S.E.V., A.D., H.H. and A.S.B prepared the first draft, reviewed and edited the manuscript. All authors read and approved the final manuscript and take responsibility for its content.

Inclusion and diversity statement

We worked to ensure gender balance in the study arms, recruited participants from diverse ethnic and socioeconomic backgrounds, and made the study questionnaire and stool collection protocol available in multiple languages.

Additional information

Supplementary Information is available for this paper. Correspondence and requests for materials should be addressed to Ami S. Bhatt (269 Campus Dr, CCSR 1155b, Stanford University, Palo Alto, CA 94305. Tel: (650) 498-4438; Email: asbhatt@stanford.edu).

Declaration of interests

The other authors declare no competing interests.

Figure legends

Figure 1. Summary of study protocol and cohort demographics

A. Sample and data collection timeline represented in days. Day 0 marks the day of enrollment in the trial, within 72 hours of a COVID-19 diagnosis. Each sample collection event is marked by a colored dot, where orange represents a blood draw and blue an oropharyngeal (OP) swab. Additionally, clinical appointments and symptom surveys are marked by yellow and green dots, respectively. Some of these events are marked by day ranges to represent collection time frames. The symptom survey at day 0 retrospectively collected symptomatology for three weeks prior to enrollment using a single questionnaire. Symptom surveys at time points centered around days 120, 210 and 300 retrospectively collected symptomatology for one week prior to the appointment using a single questionnaire at each timepoint. Collection of stool samples and their respective day ranges are marked below the timeline. Subjects were asked to provide samples in the OMNIgene GUT collection tube (OG) and the Zymo DNA/RNA shield fecal collection tube (ZY) at six time points. **B.** Cohort characteristics. 120 participants were enrolled in the clinical trial. Participants had a COVID-19 infection of mild to moderate severity and were between the ages of 18 and 71. The age and sex distribution of the paticipants are reprented here. The x-axis separates the groups by self-reported sex, and the y-axis lists age in years. Each bar represents a range of 5 years.

Figure 2. Fecal and oropharyngeal viral gRNA measurements over time

A. Summary of viral RNA positivity rates as determined by fecal and OP samples acquired from participants enrolled in the study for a period of around 28 days. The x-axis lists time point categories since enrollment as days 3 (range 0 - 7 days), 14 (8 - 21 days) and 28 (22 - 35 days). The y-axis lists the percentage of fecal samples (brown bar) and OP samples (gray bar) that tested positive at each of the time points. Fecal positivity rates are evaluated using the logistic GEE model described in the statistical methods section, which averages over all of the sample collection methods, gene types, and technical replicates; OP positivity rates are evaluated for the swab taken within three days of the fecal sample. Each bar also marks the 95% confidence interval. Number of participants and percentage positivity are listed as numbers at the top of the plot in black and red fonts, respectively, and summarized in Data S**1**. **B.** Same as panel a, except restricted to the subset of those who participated in the extended study, and following them through all 6 time points. As before, the x-axis lists time point categories since enrollment: day 3 (range 0 - 7), 14 (8 - 21), 28 (22 - 35), 120 (75 - 165), 210 (166 - 255) and 300 (>255 days), and the y-axis lists the percentage of participants with positive fecal samples (brown bar) and OP samples (gray bar) at each of the time points, with 95% confidence intervals. Number of participants and percentage positivity are listed in black and red fonts, and summarized in Data S**1**. **C.** SARS-CoV-2 viral RNA concentration in stool samples collected in the ZY kit from participants (n = 113) with mild to moderate COVID-19 infection over a time period of 300 days from enrolment in the study. Note that the ZY kits had higher overall positivity rates than the OG kits, so positivity rates in this panel tend to be slightly larger than the numbers in the previous two panels, which average over kits and genes. Fecal viral RNA concentration was determined using RT-qPCR with primers/probes targeting the E, N1, N2, RdRP genes in the SARS-CoV-2 genome as indicated in the tab at the top of each panel. The x-axis lists time point categories since enrollment. The y-axis lists the percentage of participants with a given viral RNA concentration as indicated by the color scheme in the stacked bar plot; dark blue refers to those with no detectable viral RNA, orange to viral RNA concentrations between 0 and 1 $\log_{10}$ copies per µL, yellow between 1 and 2 $\log_{10}$ copies per µL,

green between 2 and 3 $\log_{10}$ copies per µL, and light blue over 3 $\log_{10}$ copies per µL. Number of participants per time point is listed above each bar in the stacked bar plot. **D.** Fecal viral RNA concentration in stool samples collected in the ZY kit from participants (n = 113) with mild to moderate COVID-19 infection, and assayed using RT-qPCR detecting the N1 gene (viral RNA concentration in $\log_{10}$ copies per µL) vs. time (continuous variable; x-axis). Time point categories are indicated by color scheme as yellow for day 3 (range 0 - 7), lavender for day 14 (8 - 21), red for day 28 (22 - 35), gray for day 120 (75 - 165), light blue for day 210 (166 - 255) and dark blue for day 300 (>255 days). A smoothed line generated using LOESS regression (span parameter = 0.75) and 95% confidence interval is marked in the scatter plot. Note that all viral RNA concentration measurements are expressed on a logarithmic scale by applying the transformation $\log_{10}$(viral RNA concentration+1).

Figure 3. The effect of IFN-λ on fecal viral RNA shedding

A. Percentage of participants with detectable fecal SARS-CoV-2 RNA across each of the study arms, as evaluated using the logistic GEE model described in the statistical methods section. The x-axis marks the time point in the study: day 3 (range 0 - 7), 14 (8 - 21), 28 (22 - 35). The y-axis indicates the percentage of participants with detectable fecal SARS-CoV-2 RNA. The blue bar corresponds to participants in the placebo control arm, and the orange bar corresponds to participants in the IFN-λ intervention arm. Each bar also marks the 95% confidence interval. Number of participants and percentage of participants that provided a positive stool sample are listed above each stacked bar in black and red fonts, respectively, and summarized in Data S**1**. **B.** Odds ratio comparing detectable fecal SARS-CoV-2 RNA shedding in the IFN-λ intervention arm to the placebo arm at each time point in the first month of the study. The x-axis marks the odds ratio adjusted for age, sex, collection kit type (OG or ZY) and target gene (E, N1, N2, or RdRP) (aOR). The y-axis marks the time point in the study: day 3 (range 0 - 7 days), 14 (8 - 21), 28 (22 - 35). The point marks the aOR, flanked by lines denoting the 95% confidence intervals. The red dashed vertical line at aOR = 1.0 indicates no association.

Figure 4. Association between fecal viral RNA shedding and symptoms

We present these results in the overall population, as well as stratified by the presence and absence of ongoing viral RNA shedding from the oropharynx (OP): **A.** Summary of the association between viral RNA shedding and report of a given symptom, in all participants. Shedding and symptom data from up to day 28 were included in this analysis. Adjusted odds ratios (aOR) for this association were evaluated using the logistic GEE model described in the statistical methods section, which averages over collection kits (OG and ZY), target genes (E, N1, N2, and RdRP), and technical replicates, and is adjusted for age, sex, collection kit and target gene. The x-axis indicates the adjusted odds ratio (aOR) for the presence of a given symptom. The y-axis lists symptoms divided into those associated with the GI tract and those not associated with the GI tract. The odds ratio for each symptom is indicated by the circle, and associated bars represent the 95% confidence interval. The red dashed vertical line at aOR = 1.0 indicates no association. The percent of surveys reporting each symptom is provided to the left of these bars. aOR and the 95% confidence intervals are listed to the right of the bars. Analyses where sample size was insufficient are listed as “Too few reports”. **B, C.** Identical data to panel a. where panel b. lists participants

926 with negative paired OP swabs for SARS-CoV-2 RNA, and panel c. lists participants with positive
927 paired OP swabs for SARS-CoV-2 RNA.
928

929 **Tables**

930 **Table 1. Cohort demographics and associated metadata**

	Overall	GI symptoms at enrollment		Standardized difference
		Yes	No	
n	111	54	57	-
Age, median (IQR)	36 (29 - 51)	36 (29 - 49)	37 (30 - 53)	0.05
Female, n (%)	46 (41%)	26 (48%)	20 (35%)	-0.27
BMI (kg/m^2), median (IQR)	27.7 (24.8 - 31.8)	28.2 (25.0 - 32.1)	27.4 (24.7 - 30.5)	-0.25
Race / Ethnicity, n (%)				
Hispanic	72 (65%)	38 (70%)	34 (60%)	-0.22
White	28 (25%)	12 (22%)	16 (28%)	0.13
Asian	4 (4%)	3 (6%)	1 (2%)	-0.2
Unknown	6 (5%)	1 (2%)	5 (9%)	0.31
Symptomatology				
Asymptomatic at enrollment, n (%)	8 (7%)	-	8 (14%)	0.56
Duration of symptoms in days prior to randomization, median (IQR)	5 (4 - 7)	6 (5 - 8)	5 (3 - 7)	-0.61
GI symptoms at enrollment				
Any GI symptom	54 (49%)	54 (100%)	0 (0%)	-
Abdominal pain	13.0 (12%)	13.0 (24%)	-	-0.8
Diarrhea	29.0 (26%)	29.0 (54%)	-	-1.53
Nausea	31.0 (28%)	31.0 (57%)	-	-0.8
Vomiting	5.0 (5%)	5.0 (9%)	-	-0.45
Other symptoms at enrollment				
Body aches (myalgias)	59.0 (53%)	42.0 (78%)	17.0 (30%)	-1.09

Chest pain/pressure	21.0 (19%)	15.0 (28%)	6.0 (11%)	-0.45
Chills	44.0 (40%)	32.0 (59%)	12.0 (21%)	-0.84
Cough	62.0 (56%)	38.0 (70%)	24.0 (42%)	-0.59
Decreased smell	51.0 (46%)	34.0 (63%)	17.0 (30%)	-0.7
Fatigue	68.0 (61%)	43.0 (80%)	25.0 (44%)	-0.78
Fever (> 99.5 F)	10 (9%)	4 (7%)	6 (11%)	0.11
Headache	62.0 (56%)	38.0 (70%)	24.0 (42%)	-0.59
Joint pain	36.0 (32%)	25.0 (46%)	11.0 (19%)	-0.6
Shortness of breath	28.0 (25%)	17.0 (32%)	11.0 (19%)	-0.28
Sore throat	43.0 (39%)	27.0 (50%)	16.0 (28%)	-0.46
Rash	6.0 (5%)	4.0 (7%)	2.0 (4%)	-0.17
Runny nose	24.0 (22%)	16.0 (30%)	8.0 (14%)	-0.38
Laboratory values at enrollment, median (IQR)				
Absolute lymphocyte count (cells/microliter)	1.5 (1.2 - 2.2)	1.4 (1.1 - 1.9)	1.6 (1.2 - 2.3)	0.33
Alanine aminotransferase (IU/L)	30.0 (22.0 - 48.5)	31.5 (22.0 - 47.8)	28.0 (22.0 - 50.0)	0.07
Aspartate aminotransferase (IU/L)	30.0 (25.0 - 39.0)	32.5 (26.0 - 41.0)	29.0 (24.0 - 34.0)	-0.03
Seropositivity at enrollment, n (%)	46 (41%)	22 (41%)	24 (42%)	0.03
White blood cell count (cells/microliter)	5.5 (4.2 - 7.1)	5.4 (3.8 - 7.1)	5.8 (4.7 - 7.1)	0.18

931

References

1. Merola, E., Armelao, F., and de Pretis, G. (2020). Prevalence of gastrointestinal symptoms in coronavirus disease 2019: a meta-analysis. Acta Gastroenterol. Belg. *83*, 603–615.

2. Yi, Y., Lagniton, P.N.P., Ye, S., Li, E., and Xu, R.-H. (2020). COVID-19: what has been learned and to be learned about the novel coronavirus disease. Int. J. Biol. Sci. *16*, 1753–1766.

3. Cheung, K.S., Hung, I.F.N., Chan, P.P.Y., Lung, K.C., Tso, E., Liu, R., Ng, Y.Y., Chu, M.Y., Chung, T.W.H., Tam, A.R., et al. (2020). Gastrointestinal Manifestations of SARS-CoV-2 Infection and Virus Load in Fecal Samples From a Hong Kong Cohort: Systematic Review and Meta-analysis. Gastroenterology *159*, 81–95.

4. Parasa, S., Desai, M., Thoguluva Chandrasekar, V., Patel, H.K., Kennedy, K.F., Roesch, T., Spadaccini, M., Colombo, M., Gabbiadini, R., Artifon, E.L.A., et al. (2020). Prevalence of Gastrointestinal Symptoms and Fecal Viral Shedding in Patients With Coronavirus Disease 2019: A Systematic Review and Meta-analysis. JAMA Network Open *3*, e2011335–e2011335.

5. Chertow, D., Stein, S., Ramelli, S., Grazioli, A., Chung, J.-Y., Singh, M., Yinda, C.K., Winkler, C., Dickey, J., Ylaya, K., et al. (2021). SARS-CoV-2 infection and persistence throughout the human body and brain. Research Square.

6. Mao, R., Qiu, Y., He, J.-S., Tan, J.-Y., Li, X.-H., Liang, J., Shen, J., Zhu, L.-R., Chen, Y., Iacucci, M., et al. (2020). Manifestations and prognosis of gastrointestinal and liver involvement in patients with COVID-19: a systematic review and meta-analysis. The Lancet. Gastroenterology & Hepatology *5*, 667–678.

7. Sultan, S., Altayar, O., Siddique, S.M., Davitkov, P., Feuerstein, J.D., Lim, J.K., Falck-Ytter, Y., El-Serag, H.B., and AGA Institute. Electronic address: ewilson@gastro.org (2020). AGA Institute Rapid Review of the Gastrointestinal and Liver Manifestations of COVID-19, Meta-Analysis of International Data, and Recommendations for the Consultative Management of Patients with COVID-19. Gastroenterology *159*, 320–334.e27.

8. Xu, C.L.H., Raval, M., Schnall, J.A., Kwong, J.C., and Holmes, N.E. (2020). Duration of Respiratory and Gastrointestinal Viral Shedding in Children With SARS-CoV-2: A Systematic Review and Synthesis of Data. Pediatr. Infect. Dis. J. *39*, e249–e256.

9. Wang, J.-G., Cui, H.-R., Tang, H.-B., and Deng, X.-L. (2020). Gastrointestinal symptoms and fecal nucleic acid testing of children with 2019 coronavirus disease: a systematic review and meta-analysis. Sci. Rep. *10*, 17846.

10. Brooks, E.F., and Bhatt, A.S. (2021). The gut microbiome: a missing link in understanding the gastrointestinal manifestations of COVID-19? Cold Spring Harb Mol Case Stud *7*.

11. Natarajan, A., Han, A., Zlitni, S., Brooks, E.F., Vance, S.E., Wolfe, M., Singh, U., Jagannathan, P., Pinsky, B.A., Boehm, A., et al. (2021). Standardized preservation, extraction and quantification techniques for detection of fecal SARS-CoV-2 RNA. Nat. Commun. *12*, 5753.

12. Wu, Z., and McGoogan, J.M. (2020). Characteristics of and Important Lessons From the Coronavirus Disease 2019 (COVID-19) Outbreak in China: Summary of a Report of 72 314 Cases From the Chinese Center for Disease Control and Prevention. JAMA *323*, 1239–1242.

13. Oran, D.P., and Topol, E.J. (2021). The Proportion of SARS-CoV-2 Infections That Are Asymptomatic : A Systematic Review. Ann. Intern. Med. *174*, 655–662.

14. Guo, M., Tao, W., Flavell, R.A., and Zhu, S. (2021). Potential intestinal infection and faecal-oral transmission of SARS-CoV-2. Nat. Rev. Gastroenterol. Hepatol. *18*, 269–283.

15. Zhang, Y., Chen, C., Zhu, S., Shu, C., Wang, D., Song, J., Song, Y., Zhen, W., Feng, Z., Wu, G., et al. (2020). Isolation of 2019-nCoV from a Stool Specimen of a Laboratory-Confirmed Case of the Coronavirus Disease 2019 (COVID-19). China CDC Wkly *2*, 123–124.

16. Xiao, F., Sun, J., Xu, Y., Li, F., Huang, X., Li, H., Zhao, J., Huang, J., and Zhao, J. (2020). Infectious SARS-CoV-2 in Feces of Patient with Severe COVID-19. Emerg. Infect. Dis. *26*, 1920–1922.

17. Jeong, H.W., Kim, S.-M., Kim, H.-S., Kim, Y.-I., Kim, J.H., Cho, J.Y., Kim, S.-H., Kang, H., Kim, S.-G., Park, S.-J., et al. (2020). Viable SARS-CoV-2 in various specimens from COVID-19 patients. Clin. Microbiol. Infect. *26*, 1520–1524.

18. Wang, W., Xu, Y., Gao, R., Lu, R., Han, K., Wu, G., and Tan, W. (2020). Detection of SARS-CoV-2 in Different Types of Clinical Specimens. JAMA *323*, 1843–1844.

19. Zhang, Y., Chen, C., Song, Y., Zhu, S., Wang, D., Zhang, H., Han, G., Weng, Y., Xu, J., Xu, J., et al. (2020). Excretion of SARS-CoV-2 through faecal specimens. Emerg. Microbes Infect. *9*, 2501–2508.

20. Wölfel, R., Corman, V.M., Guggemos, W., Seilmaier, M., Zange, S., Müller, M.A., Niemeyer, D., Jones, T.C., Vollmar, P., Rothe, C., et al. (2020). Virological assessment of hospitalized patients with COVID-2019. Nature *581*, 465–469.

21. Albert, S., Ruíz, A., Pemán, J., Salavert, M., and Domingo-Calap, P. (2021). Lack of evidence for infectious SARS-CoV-2 in feces and sewage. Eur. J. Clin. Microbiol. Infect. Dis.

22. Gaebler, C., Wang, Z., Lorenzi, J.C.C., Muecksch, F., Finkin, S., Tokuyama, M., Cho, A., Jankovic, M., Schaefer-Babajew, D., Oliveira, T.Y., et al. (2021). Evolution of antibody immunity to SARS-CoV-2. Nature *591*, 639–644.

23. Lin, L., Jiang, X., Zhang, Z., Huang, S., Zhang, Z., Fang, Z., Gu, Z., Gao, L., Shi, H., Mai, L., et al. (2020). Gastrointestinal symptoms of 95 cases with SARS-CoV-2 infection. Gut *69*, 997–1001.

24. Bradley, B.T., Maioli, H., Johnston, R., Chaudhry, I., Fink, S.L., Xu, H., Najafian, B., Deutsch, G., Lacy, J.M., Williams, T., et al. (2020). Histopathology and ultrastructural findings of fatal COVID-19 infections in Washington State: a case series. Lancet *396*, 320–332.

25. Xiao, F., Tang, M., Zheng, X., Liu, Y., Li, X., and Shan, H. (2020). Evidence for Gastrointestinal Infection of SARS-CoV-2. Gastroenterology *158*, 1831–1833.e3.

26. Qian, Q., Fan, L., Liu, W., Li, J., Yue, J., Wang, M., Ke, X., Yin, Y., Chen, Q., and Jiang, C. (2021). Direct Evidence of Active SARS-CoV-2 Replication in the Intestine. Clin. Infect. Dis. *73*, 361–366.

27. Britton, G.J., Chen-Liaw, A., Cossarini, F., Livanos, A.E., Spindler, M.P., Plitt, T., Eggers, J., Mogno, I., Gonzalez-Reiche, A.S., Siu, S., et al. (2021). Limited intestinal inflammation despite diarrhea, fecal viral RNA and SARS-CoV-2-specific IgA in patients with acute COVID-19. Sci. Rep. *11*, 13308.

28. Cholankeril, G., Podboy, A., Aivaliotis, V.I., Tarlow, B., Pham, E.A., Spencer, S.P., Kim, D., Hsing, A., and Ahmed, A. (2020). High Prevalence of Concurrent Gastrointestinal Manifestations in Patients With Severe Acute Respiratory Syndrome Coronavirus 2: Early Experience From California. Gastroenterology *159*, 775–777.

29. Effenberger, M., Grabherr, F., Mayr, L., Schwaerzler, J., Nairz, M., Seifert, M., Hilbe, R., Seiwald, S., Scholl-Buergi, S., Fritsche, G., et al. (2020). Faecal calprotectin indicates intestinal inflammation in COVID-19. Gut *69*, 1543–1544.

30. Lamers, M.M., Beumer, J., van der Vaart, J., Knoops, K., Puschhof, J., Breugem, T.I., Ravelli, R.B.G., Paul van Schayck, J., Mykytyn, A.Z., Duimel, H.Q., et al. (2020). SARS-CoV-2 productively infects human gut enterocytes. Science *369*, 50–54.

31. Zang, R., Gomez Castro, M.F., McCune, B.T., Zeng, Q., Rothlauf, P.W., Sonnek, N.M., Liu, Z., Brulois, K.F., Wang, X., Greenberg, H.B., et al. (2020). TMPRSS2 and TMPRSS4 promote SARS-CoV-2 infection of human small intestinal enterocytes. Sci Immunol *5*.

32. Jang, K.K., Kaczmarek, M.E., Dallari, S., Chen, Y.-H., Tada, T., Axelrad, J., Landau, N.R., Stapleford, K.A., and Cadwell, K. (2022). Variable susceptibility of intestinal organoid-derived monolayers to SARS-CoV-2 infection. bioRxiv, 2021.07.16.452680.

33. Hoffmann, M., Kleine-Weber, H., Schroeder, S., Krüger, N., Herrler, T., Erichsen, S., Schiergens, T.S., Herrler, G., Wu, N.-H., Nitsche, A., et al. (2020). SARS-CoV-2 Cell Entry Depends on ACE2 and TMPRSS2 and Is Blocked by a Clinically Proven Protease Inhibitor. Cell *181*, 271–280.e8.

34. Saif, L.J., and Jung, K. (2020). Comparative Pathogenesis of Bovine and Porcine Respiratory Coronaviruses in the Animal Host Species and SARS-CoV-2 in Humans. J. Clin. Microbiol. *58*.

35. Nalbandian, A., Sehgal, K., Gupta, A., Madhavan, M.V., McGroder, C., Stevens, J.S., Cook, J.R., Nordvig, A.S., Shalev, D., Sehrawat, T.S., et al. (2021). Post-acute COVID-19 syndrome. Nat. Med. *27*, 601–615.

36. Jagannathan, P., Andrews, J.R., Bonilla, H., Hedlin, H., Jacobson, K.B., Balasubramanian, V., Purington, N., Kamble, S., de Vries, C.R., Quintero, O., et al. (2021). Peginterferon Lambda-1a for treatment of outpatients with uncomplicated COVID-19: a randomized placebo-controlled trial. Nat. Commun. *12*, 1967.

37. Lim, M.Y., Hong, S., Kim, B.-M., Ahn, Y., Kim, H.-J., and Nam, Y.-D. (2020). Changes in microbiome and metabolomic profiles of fecal samples stored with stabilizing solution at room temperature: a pilot study. Sci. Rep. *10*, 1789.

38. Coryell, M.P., Iakiviak, M., Pereira, N., Murugkar, P.P., Rippe, J., Williams, D.B., Heald-Sargent, T., Sanchez-Pinto, L.N., Chavez, J., Hastie, J.L., et al. (2021). A method for detection of SARS-CoV-2 RNA in healthy human stool: a validation study. Lancet Microbe *2*, e259–e266.

39. Kuypers, J., and Jerome, K.R. (2017). Applications of Digital PCR for Clinical Microbiology. J. Clin. Microbiol. *55*, 1621–1628.

40. Verma, R., Kim, E., Martínez-Colón, G.J., Jagannathan, P., Rustagi, A., Parsonnet, J., Bonilla, H., Khosla, C., Holubar, M., Subramanian, A., et al. (2021). SARS-CoV-2 Subgenomic RNA Kinetics in Longitudinal Clinical Samples. Open Forum Infect Dis *8*, ofab310.

41. Saif, L.J. (2010). Bovine respiratory coronavirus. Vet. Clin. North Am. Food Anim. Pract. *26*, 349–364.

42. Alexandersen, S., Chamings, A., and Bhatta, T.R. SARS-CoV-2 genomic and subgenomic RNAs in diagnostic samples are not an indicator of active replication.

43. McClary-Gutierrez, J.S., Mattioli, M.C., Marcenac, P., Silverman, A.I., Boehm, A.B., Bibby, K., Balliet, M., de Los Reyes, F.L., 3rd, Gerrity, D., Griffith, J.F., et al. (2021). SARS-CoV-2 Wastewater Surveillance for Public Health Action. Emerg. Infect. Dis. *27*, 1–8.

44. Polo, D., Quintela-Baluja, M., Corbishley, A., Jones, D.L., Singer, A.C., Graham, D.W., and Romalde, J.L. (2020). Making waves: Wastewater-based epidemiology for COVID-19 - approaches and challenges for surveillance and prediction. Water Res. *186*, 116404.

45. Medema, G., Been, F., Heijnen, L., and Petterson, S. (2020). Implementation of environmental surveillance for SARS-CoV-2 virus to support public health decisions: Opportunities and challenges. Curr Opin Environ Sci Health *17*, 49–71.

46. Mallapaty, S. (2020). How sewage could reveal true scale of coronavirus outbreak. Nature *580*, 176–177.

47. CDC (2022). National Wastewater Surveillance System (NWSS). Centers for Disease Control and Prevention. https://www.cdc.gov/healthywater/surveillance/wastewater-surveillance/wastewater-surveillance.html?CDC_AA_refVal=https%3A%2F%2Fwww.cdc.gov%2Fcoronavirus%2F2019-ncov%2Fcases-updates%2Fwastewater-surveillance.html.

48. Mowat, A.M., and Agace, W.W. (2014). Regional specialization within the intestinal immune system. Nat. Rev. Immunol. *14*, 667–685.

49. Osikomaiya, B., Erinoso, O., Wright, K.O., Odusola, A.O., Thomas, B., Adeyemi, O., Bowale, A., Adejumo, O., Falana, A., Abdus-Salam, I., et al. (2021). "Long COVID": persistent COVID-19 symptoms in survivors managed in Lagos State, Nigeria. BMC Infect. Dis. *21*, 304.

50. Taquet, M., Dercon, Q., Luciano, S., Geddes, J.R., Husain, M., and Harrison, P.J. (2021). Incidence, co-occurrence, and evolution of long-COVID features: A 6-month retrospective cohort study of 273,618 survivors of COVID-19. PLoS Med. *18*, e1003773.

51. Ramakrishnan, R.K., Kashour, T., Hamid, Q., Halwani, R., and Tleyjeh, I.M. (2021). Unraveling the Mystery Surrounding Post-Acute Sequelae of COVID-19. Front. Immunol. *12*, 686029.

52. Weng, J., Li, Y., Li, J., Shen, L., Zhu, L., Liang, Y., Lin, X., Jiao, N., Cheng, S., Huang, Y., et al. (2021). Gastrointestinal sequelae 90 days after discharge for COVID-19. Lancet Gastroenterol Hepatol *6*, 344–346.

53. Carfì, A., Bernabei, R., Landi, F., and Gemelli Against COVID-19 Post-Acute Care Study Group (2020). Persistent Symptoms in Patients After Acute COVID-19. JAMA *324*, 603–605.

54. Kayaaslan, B., Eser, F., Kalem, A.K., Kaya, G., Kaplan, B., Kacar, D., Hasanoglu, I., Coskun, B., and Guner, R. (2021). Post-COVID syndrome: A single-center questionnaire study on 1007 participants recovered from COVID-19. J. Med. Virol. *93*, 6566–6574.

55. Al-Aly, Z., Xie, Y., and Bowe, B. (2021). High-dimensional characterization of post-acute sequelae of COVID-19. Nature *594*, 259–264.

56. Lu, X., Wang, L., Sakthivel, S.K., Whitaker, B., Murray, J., Kamili, S., Lynch, B., Malapati, L., Burke, S.A., Harcourt, J., et al. (2020). US CDC Real-Time Reverse Transcription PCR Panel for Detection of Severe Acute Respiratory Syndrome Coronavirus 2. Emerg. Infect. Dis. *26*.

57. Droplet Digital PCR Applications Guide https://www.bio-rad.com/webroot/web/pdf/lsr/literature/Bulletin_6407.pdf.

58. dMIQE Group, and Huggett, J.F. (2020). The Digital MIQE Guidelines Update: Minimum Information for Publication of Quantitative Digital PCR Experiments for 2020. Clin. Chem. *66*, 1012–1029.

59. Austin, P.C. (2009). Using the Standardized Difference to Compare the Prevalence of a Binary Variable Between Two Groups in Observational Research. Communications in Statistics - Simulation and Computation *38*, 1228–1234.

60. Cohen, J. (1988). The effect size index: d. Statistical power analysis for the behavioral sciences. Abingdon-on-Thames: Routledge Academic.

61. Liang, K.-Y., and Zeger, S.L. (1986). Longitudinal data analysis using generalized linear models. Biometrika *73*, 13–22.

62. Kernan, W.N., Viscoli, C.M., Makuch, R.W., Brass, L.M., and Horwitz, R.I. (1999). Stratified randomization for clinical trials. J. Clin. Epidemiol. *52*, 19–26.

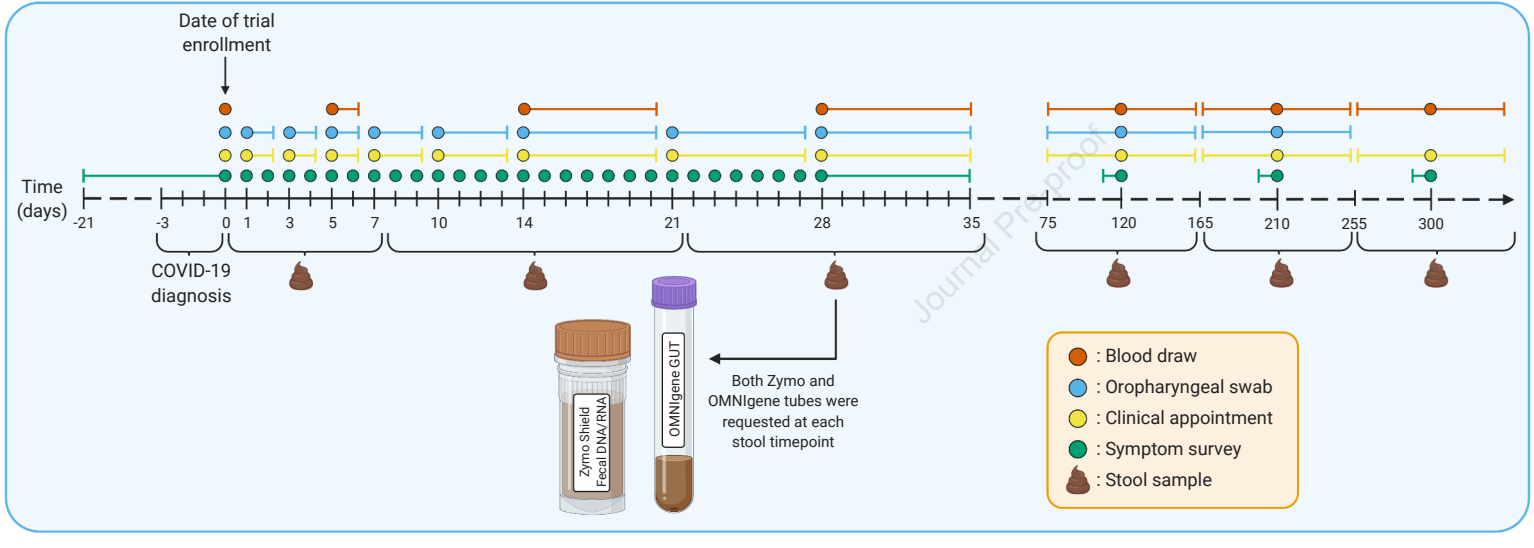
63. Pierson-Perry, J.F., Vaks, J.E., Vore, T.E.K., Durham, A.P., Fischer, C., Gutenbrunner, C., Hillyard, D., Kondratovich, M.V., Ladwig, P., and Middleberg, R.A. (2012). Evaluation of detection capability for clinical laboratory measurement procedures; approved guideline (Clinical Laboratory Standards Institute).

64. Corman, V.M., Landt, O., Kaiser, M., Molenkamp, R., Meijer, A., Chu, D.K., Bleicker, T., Brünink, S., Schneider, J., Schmidt, M.L., et al. (2020). Detection of 2019 novel coronavirus (2019-nCoV) by real-time RT-PCR. Euro Surveill. *25*.

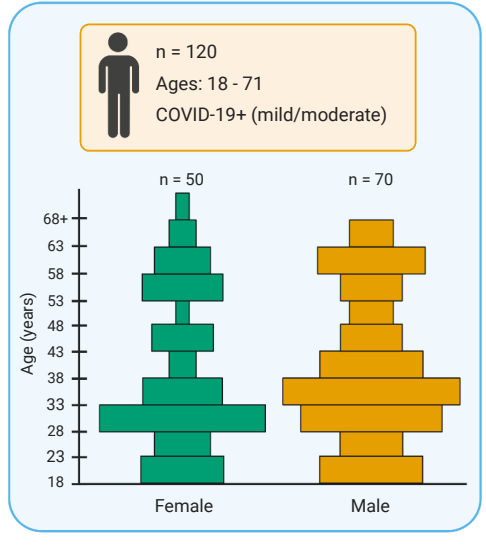
65. CDC 2019-Novel Coronavirus (2019-nCoV) Real-Time RT-PCR Diagnostic Panel (2020). (Division of Viral Diseases, Centers for Disease Control and Prevention).

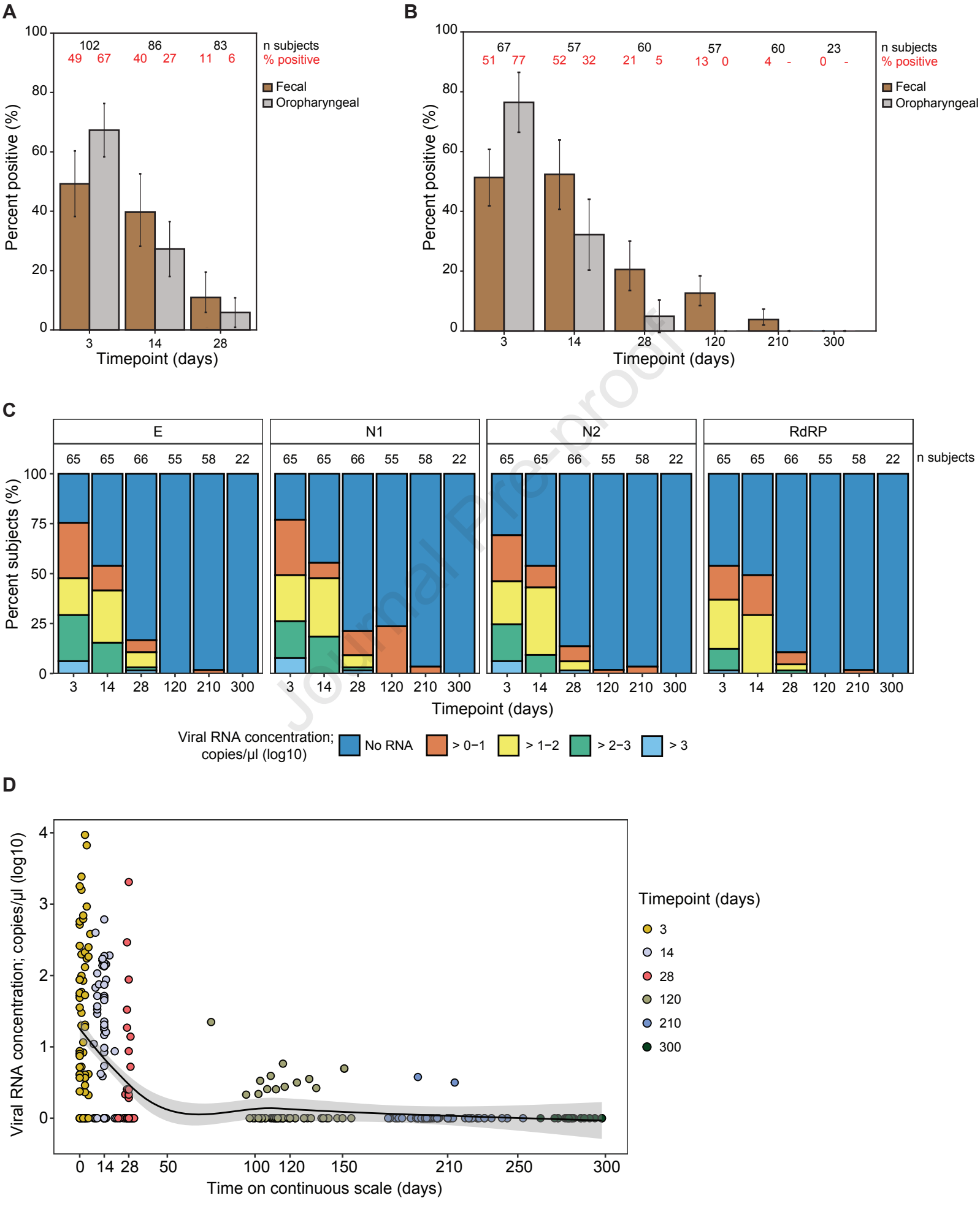
66. Bustin, S.A., Benes, V., Garson, J.A., Hellemans, J., Huggett, J., Kubista, M., Mueller, R., Nolan, T., Pfaffl, M.W., Shipley, G.L., et al. (2009). The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin. Chem. *55*, 611–622.

A Sample and data collection timeline

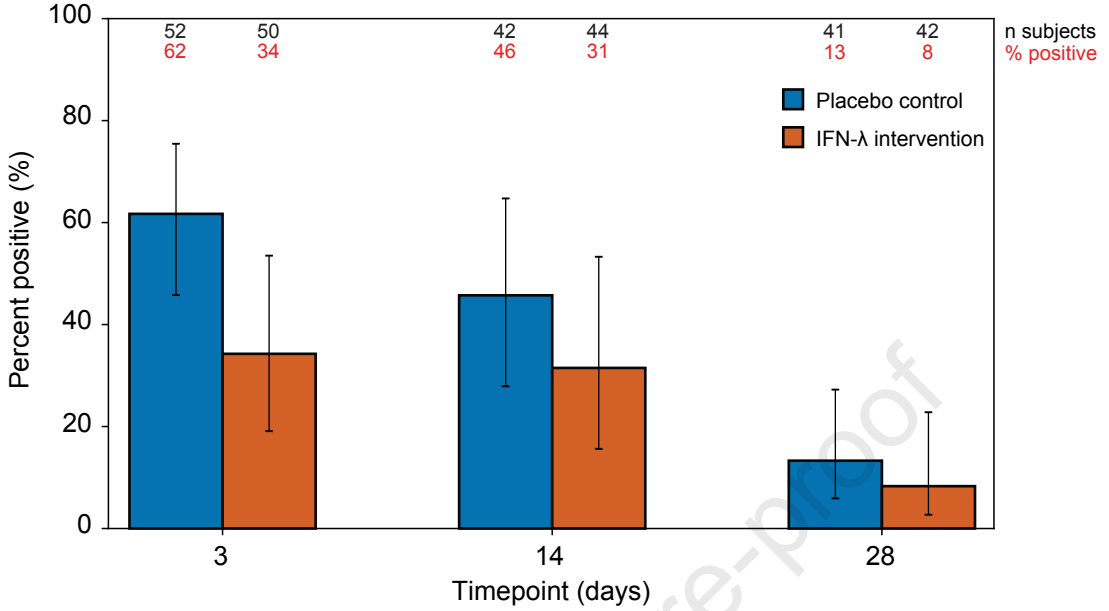


B Cohort characteristics

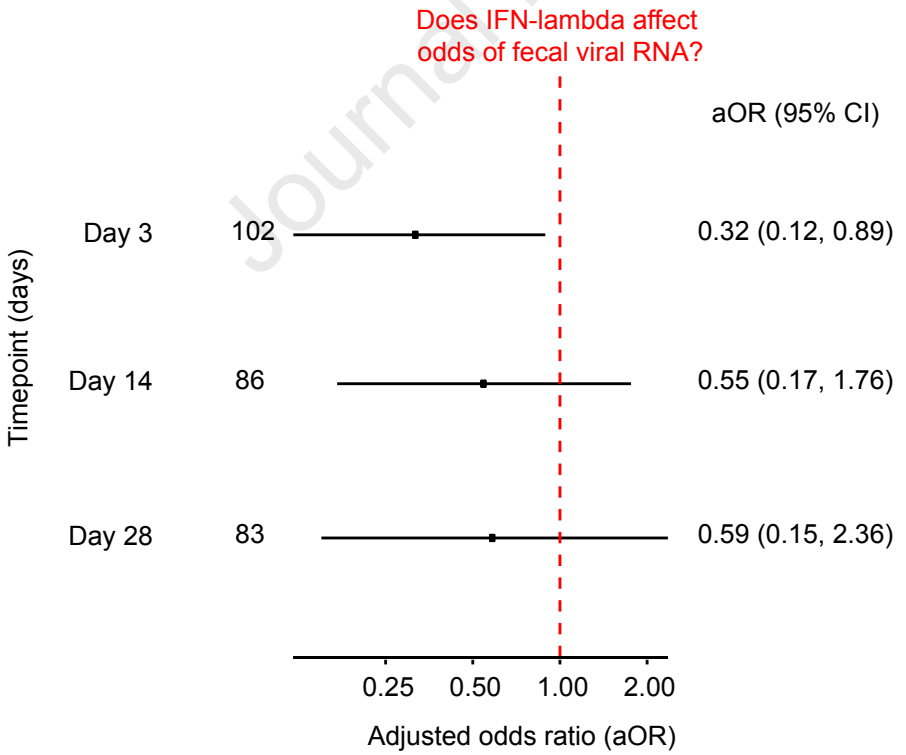


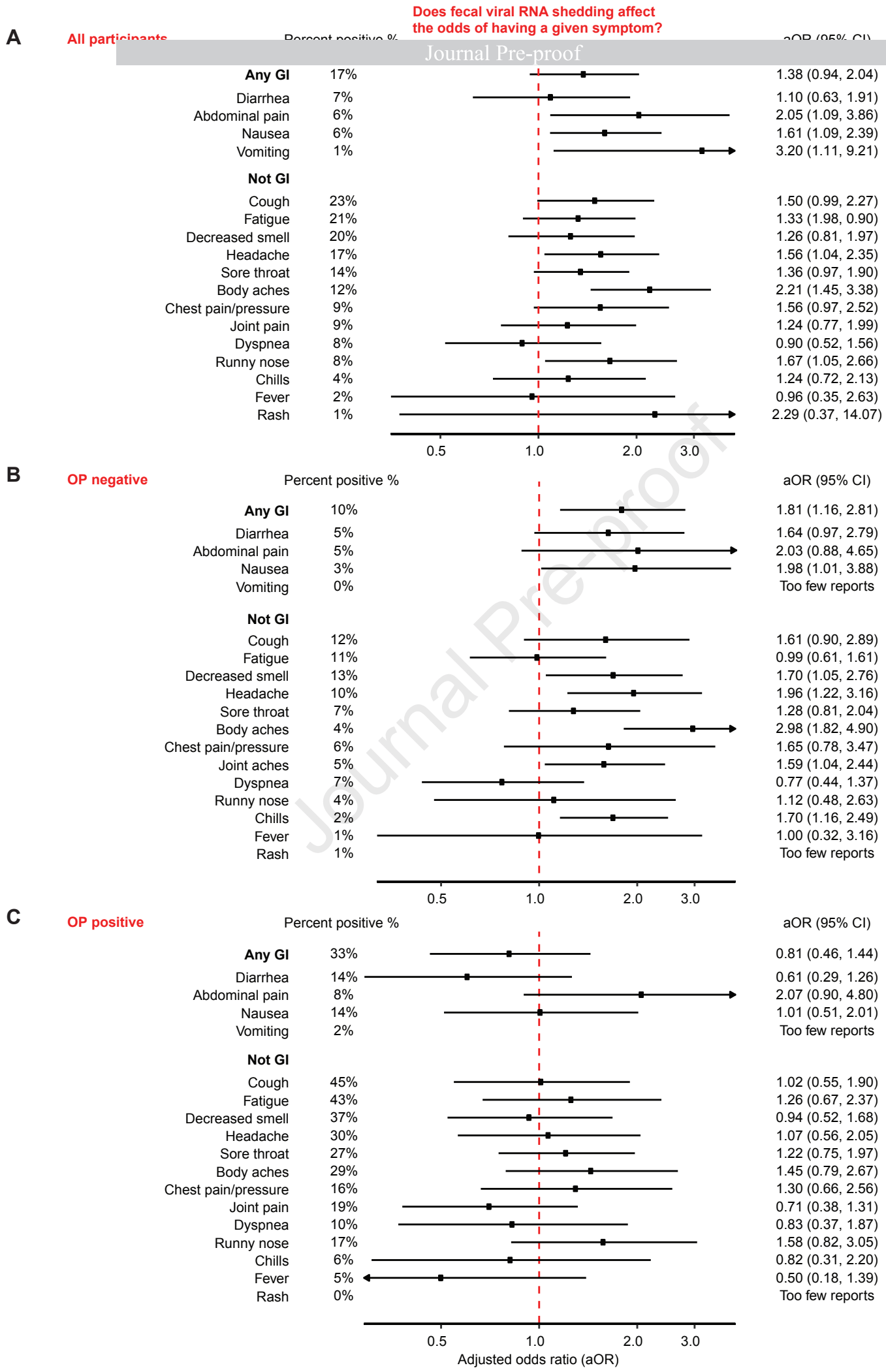


A



B





Title

Gastrointestinal symptoms and fecal shedding of SARS-CoV-2 RNA suggest prolonged gastrointestinal infection

Authors

Aravind Natarajan[1,2,*], Soumaya Zlitni[1,2,*], Erin F. Brooks[2,*], Summer E. Vance[2,*], Alex Dahlen[3], Haley Hedlin[3], Ryan M. Park, Alvin Han[4], Danica T. Schmidtke[4], Renu Verma[5], Karen B. Jacobson[5], Julie Parsonnet[6,7], Hector F. Bonilla[6], Upinder Singh[5], Benjamin A. Pinsky[5,8], Jason R. Andrews[5], Prasanna Jagannathan[4,6], Ami S. Bhatt^[1,2]

Context and Significance

Gastrointestinal symptoms and SARS-CoV-2 RNA shedding in feces point to the gastrointestinal tract as a possible site of infection in COVID-19. Researchers from Stanford University measured the dynamics over time of fecal viral material in patients with mild to moderate COVID-19 followed for 10 months post-diagnosis. The authors found that fecal viral RNA shedding was correlated with gastrointestinal symptoms in patients who had cleared their respiratory infection. They also observed fecal shedding can continue to 7 months post-diagnosis In conjunction with recent related findings, this work presents compelling evidence of SARS-CoV-2 infection in the gastrointestinal tract and suggests a possible role for long-term infection of the gastrointestinal tract in syndromes such as “long COVID”.

eTOC blurb

Natarajan et al perform a longitudinal study of fecal SARS-CoV-2 RNA shedding in patients with mild-to-moderate COVID-19, revealing that patients can shed RNA for up to 7 months after infection, shedding is associated with gastrointestinal symptoms, and the gastrointestinal tract may be infected even after the respiratory infection has cleared.

Highlights

1. Approximately half of COVID-19 patients shed fecal RNA in the week after infection
2. 4% patients with COVID-19 shed fecal viral RNA 10 months after diagnosis.
3. Presence of fecal SARS-CoV-2 RNA is associated with gastrointestinal symptoms.
4. SARS-CoV-2 likely infects gastrointestinal tissue.

Key resources table

Reagent or resource	Source	Identifier
Biological Samples		
Stool from participants in Peginterferon Lambda-1a (IFN-λ) clinical trial (NCT04331899)	Stanford University	N/A
Oropharyngeal swabs from participants in Peginterferon Lambda-1a (IFN-λ) clinical trial (NCT04331899)	Stanford University[36]	N/A
Chemicals, Peptides, and Recombinant Proteins		
Phosphate buffered saline (PBS)	Fisher Scientific	BP399-500
0.8 mM Ethylenediaminetetraacetic Acid (EDTA)	Fisher Scientific	EC200-449-9
Nuclease-free water	Ambion	AM9937
Tris-HCl pH 8.0	Invitrogen	15567-027
Critical Commercial Assays		
QiaAMP Viral RNA Mini kit	Qiagen	52906
Custom ddPCR Assay Primer/Probe Mix	BioRad	10031277
One-Step RT-ddPCR Advanced Kit for Probes	BioRad	1864021
TaqPath 1-Step RT-qPCR Master Mix, CG	ThermoFisher	A15299
Deposited Data		
A digital repository of all data supporting the findings of this study can be found at Zenodo	This study	https://zenodo.org/record/6374138
Oligonucleotides		

Primers for RT-qPCR and ddPCR used in this study, see Table S6	This study	N/A
Probes for RT-qPCR and ddPCR used in this study, see Table S6	This Study	N/A
Recombinant DNA		
Synthetic SARS-CoV-2 RNA	ATCC	VR-3276SD
Zoetis Calf-Guard Bovine Rotavirus-Coronavirus Vaccine	Zoetis	VLN 190/PCN 1931.20
Software and Algorithms		
Design and Analysis software	Thermo Fisher Scientific	Version 2.5.1
REDCap Cloud	https://projectredcap.org/	Version 1.5
Python	https://www.python.org/	Version 3.8.5
Statsmodel package	https://www.statsmodels.org/stable/index.html	Version 0.12.0
RStudio	https://www.rstudio.com/	Version 1.3.959
Other		
Biomek-FX liquid handler	Biomek	N/A
12k Flex Applied Biosystems qPCR machine	Applied Biosystems	N/A
QX200 AutoDG Droplet Digital PCR System	BioRad	N/A
BioRad C1000 thermocycler	BioRad	N/A
ddPCR reader	BioRad	QX200
OMNIGene GUT collection tube	DNA Genotek	OM-200
Toilet accessory	DNA Genotek	OM-AC1
DNA/RNA shield fecal collection tube	Zymo	R1101-E
96-well plates	BioRad	HSP9601

Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines, see Table S7 and Table S8	dMIQE Group & Huggett et al. 2020	Digital MIQE guidelines
Droplet Digital PCR Applications Guide on QX200 machines	BioRad	Droplet Digital PCR Applications Guide
MicroAmp Optical 384-well plates	FisherScientific	43-098-49
Optically clear seal	Applied biosystems	4311971