

# Clinical and In Vitro Evidence Favoring Immunoglobulin Treatment of a Chronic Norovirus Infection in a Patient With Common Variable Immunodeficiency

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**Background.** Immunocompromised individuals can become chronically infected with norovirus, but effective antiviral therapies are not yet available.

**Methods.** Treatments with nitazoxanide, ribavirin, interferon alpha-2a, and nasoduodenally administered immunoglobulins were evaluated sequentially in an immunocompromised patient chronically infected with norovirus. In support, these components were also applied to measure norovirus inhibition in intestinal enteroid cultures in vitro. Viral RNA levels were determined in fecal and plasma samples during each treatment and viral genomes were sequenced.

**Results.** None of the antivirals resulted in a reduction of viral RNA levels in feces or plasma. However, during ribavirin treatment, there was an increased accumulation of virus genome mutations. In vitro, an effect of interferon alpha-2a on virus replication was observed and a genetically related strain was neutralized effectively in vitro using immunoglobulins and post-norovirus-infection antiserum. In agreement, after administration of immunoglobulins, the patient cleared the infection.

**Conclusions.** Intestinal enteroid cultures provide a relevant system to evaluate antivirals and the neutralizing potential of immunoglobulins. We successfully treated a chronically infected patient with immunoglobulins, despite varying results reported by others. This case study provides in-depth, multifaceted exploration of norovirus treatment that can be used as a guidance for further research towards norovirus treatments.

**Keywords.** immunocompromised; ribavirin; nitazoxanide; interferon alpha-2a; immunoglobulins; norovirus; next-generation sequencing; VirCapSeq; enteroids; human intestinal organoids.

There is a growing number of immunocompromised patients due to increased solid organ transplantation, increased incidence of cancer, and advances in the care of patients with primary immunodeficiency diseases (PID) [1]. Norovirus is the most common viral cause of gastroenteritis and these infections can become chronic in immunocompromised individuals [2]. The mechanisms behind viral clearance are not clear, although high titers of immunoglobulin (Ig) G antibodies that can block binding of the virus to its receptors correlate with protection [3]. Norovirus-specific IgA is associated with

protection and a shorter duration of virus shedding [4], and norovirus-specific T cells have been detected in children and blood donors [5, 6]. There are at least 10 norovirus genogroups that can be further subdivided in genotypes, and as a result individual can be infected multiple times during their lifetime [7, 8]. Most infections are caused by genotype GII.4, for which new variants that can escape herd immunity emerge over time [9]. It is, therefore, likely that people have a broad antibody response against GII.4.

In patients that receive immunosuppressive therapy, tapering of these drugs may be used to clear norovirus infections [10], but this is not possible for PID patients and no licensed vaccines or therapeutic drugs are currently available for treatment [11]. Both ribavirin and nitazoxanide have been used off label to treat norovirus infections [12–14]. Ribavirin is a nucleoside analogue and is used as an antiviral treatment for chronic hepatitis C infections. Nitazoxanide is a broad-spectrum antiparasitic drug, and has been studied as a broad-spectrum antiviral. Nitazoxanide inhibited norovirus replication in a replicon system [15]. Another potential candidate is interferon alpha, which reduced norovirus shedding in pigs [16]. Ig therapies are

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used to treat various infections in PID patients. Such Ig preparations are manufactured from plasma collected from a large number of donors to ensure diverse specificities of antibodies against a broad spectrum of pathogens [17]. In patients chronically infected with norovirus, Ig have been applied directly to the gastrointestinal tract [18–20]. All treatments described above have been used empirically with varying success rates, with some patients rapidly being cured and others not. Detailed investigations of the viral genotypes, shedding patterns, and clearance mechanisms throughout the course of treatment(s) were generally lacking in these studies, making it difficult to assess why the outcomes of treatment varied and how therapy may be improved. Importantly, the absence of a norovirus cell culture model has previously complicated the development of antivirals, but two such models have now become available, one based on B cells and the other on human intestinal enteroids [21–23], which may aid the rational optimization of treatment of chronic norovirus infection.

Here we describe a patient with common variable immune deficiency (CVID) suffering from a debilitating chronic norovirus infection, who was sequentially treated with nitazoxanide, ribavirin, interferon alpha-2a, and nasoduodenally administered Ig. To corroborate treatment effects, we performed in-depth monitoring of clinical response, viral loads, and inpatient norovirus evolution before, during, and after the sequential treatments. In support, the same treatments were evaluated in intestinal enteroids *in vitro*. Both *in vitro* and *in vivo*, only the administration of Ig resulted in effective viral clearance.

## METHODS

### Norovirus Quantification

Feces, plasma, and samples from the *in vitro* norovirus replication assays were tested with a semiquantitative real-time polymerase chain reaction assay (RT-qPCR) with primers and probes used in the routine molecular viral diagnostics setting of Erasmus Medical Center as described previously [24–26]. Changes larger than 3 cycle threshold (Ct) values, which corresponds to 1 log difference in genome equivalents, were considered significant. Samples were stored at  $-80^{\circ}\text{C}$ .

### Next-Generation Sequencing

Clarified 10% (w/v) fecal suspension or EDTA plasma were pretreated with OmniCleave Endonuclease (Lucigen) to remove host nucleic acids (NA); viral RNA (vRNA) in intact particles is not affected by this treatment. RNA was extracted using a Viral RNA Mini Kit (Qiagen) and subjected to whole-genome sequencing using VirCapSeq-VERT for viral enrichment as described previously [27, 28]. In short, double-stranded DNA synthesis was performed with random primers, Superscript IV (ThermoFisher Scientific) and Klenow (New England Biolabs). The KAPA HyperPlus library preparation

kit (Roche) was used according to the manufacturer's instructions with a shorter shearing time of 3 minutes and adapters diluted 1:10. After adapter ligation, an additional AMPure bead step was performed before capture. Sequencing was performed on an Illumina MiSeq using the MiSeq Reagent Kit version 3 (Illumina). Adapters were removed and sequence reads were trimmed to a PHRED score of 33 using trimmomatic-0.38 [29]. Resulting reads were *de novo* assembled using SPAdes version 3.11.0 and classified using DIAMOND [30]. Contigs were further assembled into genomes using Geneious version 9.1.8, genotyped using the noronet genotyping tool [31], and submitted to the National Center for Biotechnology Information (NCBI): MW581004 (day 1), MW581005 (day 168), MW581006 (day 182), MW581007 (day 274), MW581008 (day 335), MW581009 (day 359), MW581010 (day 384), MW581011 (day 421), and MW581012 (day 447). The norovirus genome has 3 open reading frames (ORFs), encoding a polyprotein, including the polymerase (ORF1), the viral capsid (VP1, ORF2), and viral protein 2 (ORF3). VP1 sequences were aligned with GII.4 Orleans 2009 VP1 sequences obtained from NCBI. Phylogenies were inferred using IQ-tree software under the Transition Model (TIM2e) + R4 [32] and visualized using Figtree [33].

### Cell Culture and Generation of Enteroid Monolayers for Norovirus Infection

The secretor-positive jejunal J2 human intestinal enteroids were maintained as previously described [23, 34]. For infections, monolayer cultures in 96-well plates were prepared as described previously [23]. In short, plates were coated with collagen from human placenta (Sigma-Aldrich). The enteroid cultures were washed with 0.5 mM EDTA in ice-cold phosphate-buffered saline (PBS), without  $\text{CaCl}_2$  and  $\text{MgCl}_2$ , and dissociated with 0.05% trypsin/0.5 mM EDTA for 4 minutes at  $37^{\circ}\text{C}$ , trypsin was inactivated by adding 10% fetal bovine serum diluted in complete medium without growth factors (CMGF $-$ ). Next, cells were passed through a 40- $\mu\text{m}$  cellstrainer and seeded in 96-well plates in complete medium with growth factors (CMGF $+$ ) containing ROCK inhibitor (10  $\mu\text{M}$ , Sigma-Aldrich). After 24 hours, the CMGF $+$  was replaced with differentiation medium to allow differentiation for 4 days [23].

### Evaluation of Antivirals in the Norovirus Culture Model

Nitazoxanide (Sigma-Aldrich) and ribavirin were dissolved in dimethyl sulfoxide (DMSO), and interferon alpha-2a in PBS, and stored at  $-20^{\circ}\text{C}$  until use. Norovirus-positive stool suspensions were prepared by adding PBS to the stools, homogenizing by vortexing, and clarification at 1500g for 15 minutes at  $4^{\circ}\text{C}$ . For the evaluation of antivirals, a norovirus-positive stool suspension containing the same genotype as that of the patient, a GII.4\_Sydney\_2012 [31] strain, was used. The stool was first diluted in CMGF $-$  with 500  $\mu\text{M}$  sodium glycochenodeoxycholate (GCDCA; Sigma-Aldrich). Next, the J2 human intestinal

enteroid monolayers in 96-well plates were inoculated with  $1 \times 10^6$  genome equivalents for 2 hours at 37°C to allow attachment of the virus to the cells. Directly after inoculation, the cells were washed 3 times with Advanced DMEM/F12 (Invitrogen) and the medium was replaced with differentiation medium with GCDCA (Sigma-Aldrich) containing the antivirals nitazoxanide (Sigma-Aldrich), ribavirin (Sigma-Aldrich), DMSO, or interferon alpha-2a (Sigma-Aldrich). All conditions were performed in duplicate. At 0 and 72 hours postinoculation cells and supernatant were collected and stored at -80°C prior to RNA isolation. RNA was isolated using the High Pure Viral Nucleic Acid Kit (Roche). Levels of vRNA were quantified by RT-qPCR. A standard curve based on a norovirus RNA transcript was used to quantitate viral genome equivalents in RNA samples.

#### Virus Neutralization Assays

For the neutralization assay, the GII.4\_Sydney\_2012 [31] strain was again used. Two-fold dilution serum dilutions starting at 1:40 were prepared. Each serum dilution was mixed with an equal volume of differentiation medium containing  $1 \times 10^6$  genome equivalents and GCDCA (Sigma-Aldrich). The mixture was incubated for 1 hour at 37°C. Following this incubation, the virus serum mixture was transferred to the differentiated J2 enteroid monolayers in 96-well plates and inoculated for 2 hours at 37°C before washing and replacing the media with differentiation medium containing GCDCA. At 0 and 72 hours postinoculation, samples of all infection experiments were placed at -80°C until further analysis. Levels of vRNA were quantified by RT-qPCR. A standard curve based on human norovirus RNA transcript was used to quantitate viral genome equivalents in RNA samples.

#### Ethics

The use of patient samples and enteroids was approved by the Erasmus Medical Center ethical committee under METC-2018-1307. The patient signed informed consent and the study was approved by the Erasmus Medical Center ethical committee under METC-2013-026.

## RESULTS

#### Case Presentation

A female patient was diagnosed with CVID in 2013 at age 39. She had a body mass index (BMI) of 20.8 kg/m<sup>2</sup> and presented with a history of recurrent upper and lower respiratory tract infections and suffered from a *Giardia lamblia* gastroenteritis in 2012. Immunological evaluation revealed low IgG (1.7 g/L; reference 7.0–16.0 g/L), low IgA (<0.01 g/L; reference 0.76–3.91 g/L), low IgM (<0.30 g/L; reference 0.45–2.3 g/L), and an absent response after vaccination with polysaccharide and polysaccharide-protein conjugate vaccines. Total B lymphocyte numbers were within normal limits, but the absolute number

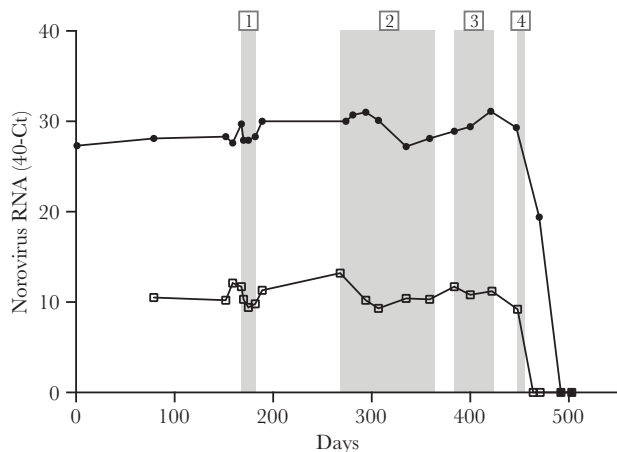
of memory B lymphocytes was low (7 cells/μL; reference 13–122 cells/μL). There were no signs of autoimmune disease, autoinflammatory complications, or hematological malignancy. She was treated with intravenous Ig replacement therapy (IGRT), 25 grams every 4 weeks since September 2013, which resulted in IgG trough levels of around 8.5 g/L. In November 2015, she commenced on facilitated subcutaneous IGRT, 25 grams every 4 weeks and this was maintained throughout the course of treatment described here. In 2018, whole-exome sequencing was performed to evaluate the presence of genetic variants in PID-associated genes, but no pathogenic variants were detected.

December 2016, she presented with symptoms of a gastrointestinal tract infection and stool was found positive for norovirus. Initially, dosing of IGRT was gradually increased to a dose of 40 grams every 4 weeks (0.8 g/kg body weight). However, the patient remained symptomatic with diarrhea, 2–7 times per day, without macroscopic blood loss, resulting in 2-kilogram weight loss over 3 months and a BMI of 20 kg/m<sup>2</sup>. Feces and plasma samples remained positive for norovirus vRNA and sequence analysis revealed a chronic infection with a GII.4 New Orleans 2009[P4] strain. RT-PCR showed approximately  $1 \times 10^{10}$  genome equivalents/mL in feces and 6 logs lower in plasma (Figure 1). Persisting symptoms, including progressive weight loss, necessitated consideration of off-label treatment with antivirals, which was extensively discussed with the patient and consent was provided.

#### Norovirus Infection Treatment Strategies

In August 2017, treatment with oral nitazoxanide was initiated, 500 mg twice daily for 14 days. Initially, once a day normal stool was reported, amongst 3–5 episodes of diarrhea per day. However, after 2 weeks there was no further clinical improvement or decrease in vRNA levels (Figure 1). Therefore, a second treatment regimen of oral ribavirin, 400 mg twice daily, was started in December 2017. This treatment was complicated by adverse effects (fatigue and loss of appetite) and did not result in clinical improvement or a substantial decline in vRNA levels. Because of persisting diarrhea, 2–4 times per day, her BMI declined to 18 kg/m<sup>2</sup> in March 2018 and treatment with ribavirin was stopped.

A third treatment, interferon alpha-2a (Roferon-A), was administered as subcutaneous injections of  $3 \times 10^6$  units 3 times per week. Because of her BMI (17.5 kg/m<sup>2</sup>) she was also started on an energy- and protein-enriched diet. After 1 month of therapy there was no clinical improvement and no decline in vRNA levels. Because of progressive weight loss (BMI = 16 kg/m<sup>2</sup>) tube feeding was commenced in April 2018. After 1 month the patient still suffered from diarrhea (2–4 times per day) resulting in a BMI of 15.9 kg/m<sup>2</sup> and vRNA levels remained high. As final rescue therapy, in May 2018, 25 mg/kg Ig (Privigen [35]) was administered nasoduodenally, 4 times daily for 2



**Figure 1.** The effect of anti norovirus treatment on norovirus RNA levels. Norovirus RNA levels in feces (circles) and EDTA plasma (squares) during treatment are shown as cycle threshold (Ct) 40 (the lower limit of detection) minus the Ct value. The grey areas represent the duration of treatments. Treatment 1, 14 days of oral nitazoxanide, 500 mg twice daily. Treatment 2, 96 days of oral ribavirin, 400 mg twice daily. Treatment 3, 39 days of interferon alpha-2a, subcutaneous injections of  $3 \times 10^6$  units 3 times per week. Treatment 4, 2 days of oral immunoglobulin, 25 mg/kg 4 times daily.

days. Ig was administered via a nasoduodenal tube to prevent gastric degradation. Thirteen days after treatment there was no detectable vRNA in the plasma, a significant decrease in vRNA levels in the feces, and resolution of clinical symptoms. The last day of shedding was not determined but after 45 days the patient was negative for vRNA in both feces and plasma. Her body weight gradually increased over time and her BMI returned to  $18 \text{ kg/m}^2$  within 3 months; currently her BMI is  $23.2 \text{ kg/m}^2$  and, after 18 months of consistently having diarrhea, she has remained free from symptoms of a gastrointestinal tract infection.

#### Impact of Treatment on the Viral Genome

Selective drug pressure can lead to selection of mutations that renders a virus less susceptible or resistant to treatment. Thus, monitoring of inpatient virus evolution under selective drug pressure gives information on its activity in patients and enables the dissection of virological response from clinical response in patients. In addition, certain nucleoside analogs like ribavirin exert their action via lethal mutagenesis and an increase in the accumulation of viral mutations during treatment would indicate some effect of these drugs on norovirus replication. Therefore, fecal samples were subjected to next-generation sequencing.

In this patient, vRNA was detected in the feces and plasma, and one hypothesis was that vRNA in plasma was the result of extraintestinal replication in peripheral B cells. Prolonged replication in B cells could, over time, result in distinct genomic features compared to virus replicating in the intestine. Therefore, plasma samples were included for sequencing.

Only short sequences could be obtained from the plasma. This is likely due to the low levels and potentially low quality of vRNA in the plasma. With the protocol used, only nucleic acids of largely intact viral particles would be detected. Complete genome sequences were obtained for all fecal samples except for the time point after Ig treatment. The VP1 sequences from the patient were most closely related to GII.4 New Orleans 2009, but a phylogenetic tree showed that these sequences were highly divergent from other GII.4 New Orleans 2009 VP1 sequences (Figure 2). The short sequences that were retrieved from plasma were similar to those detected in fecal samples at the same time points (data not shown).

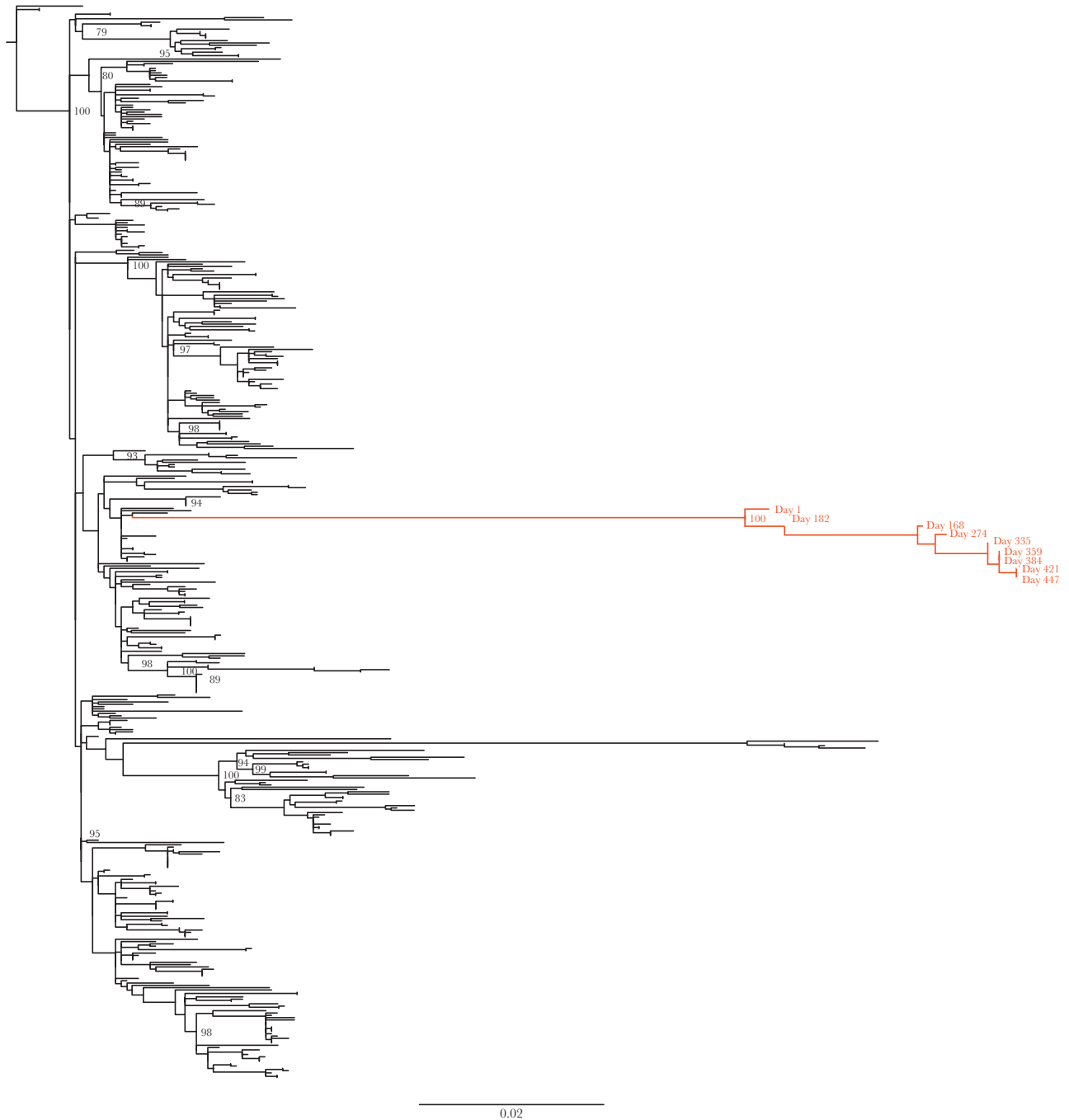
The accumulated numbers of nucleotide and amino acid substitutions in the consensus sequences are shown in Figure 3, and nucleotide and amino acid changes for individual proteins and in antigenic domains are given in Supplementary Tables 1–3 [36]. During the ribavirin treatment there was an increase of nucleotide and amino acid substitutions followed by a decrease after treatment. However, in this patient, the effect was not large enough to result in lethal mutagenesis and clearance of norovirus. The decrease of mutations after ribavirin treatment were the result of a reversion of mutations in the consensus sequence, and due to varying amounts of quasispecies that affected the consensus sequence (data not shown). Overall, there was an increase in the number of incorporated nucleotide substitutions over time, with most accumulating in ORF2 in the antigenic domains.

#### Effect of Antivirals on GII.4 Replication In Vitro

In parallel, the effect of nitazoxanide and ribavirin on norovirus replication was evaluated in vitro in human intestinal enteroid cultures. In total 7 fecal samples from the patient, obtained between August 2017 and March 2018, were used to inoculate these cultures, but none of them resulted in virus replication (data not shown). We therefore used another norovirus strain of the same genotype that replicated to high titers to evaluate the different treatment strategies. Monolayers were inoculated with a GII.4 Sydney 2012[P31] strain; directly after inoculation the antivirals were added to the medium. The monolayers remained intact for all concentrations of ribavirin, nitazoxanide, interferon alpha-2a, and DMSO except for  $100 \mu\text{M}$  nitazoxanide, for which all cells were detached after 24 hours. Both ribavirin and nitazoxanide did not inhibit norovirus replication (Figure 4A) at concentrations where the cell layer was still intact. Interferon alpha-2a inhibited GII.4 norovirus replication at  $1000 \text{ IU/mL}$  (Figure 4B).

#### Ig Neutralizes GII.4 Viruses In Vitro

Because the nasoduodenal administration of Ig to the patient ultimately led to clearance of the infection, we investigated whether Ig preparations contain norovirus-specific antibodies that can neutralize virus replication in vitro. In addition, we



**Figure 2** Maximum likelihood tree of the GII.4 New Orleans 2009 VP1 sequences. The consensus sequences of the patient are colored orange. Selected bootstrap values > 70 are depicted.

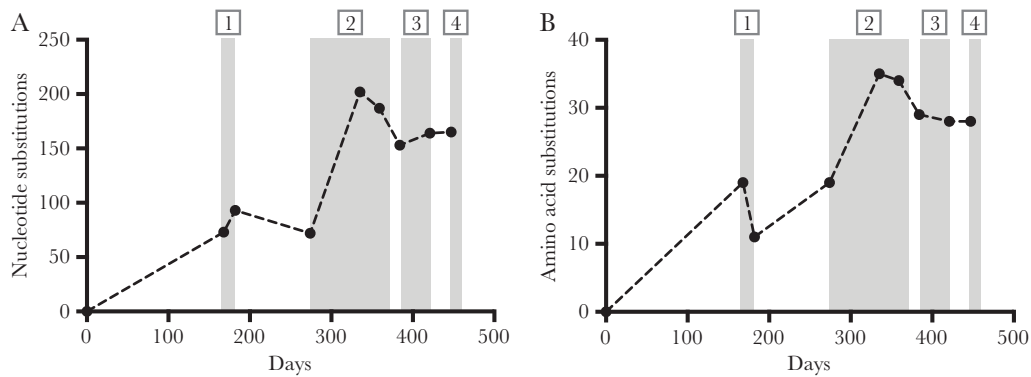
investigated how these virus neutralization titers compare to those after an acute norovirus infection. A virus neutralization assay was performed with serum and 2 batches of Ig from Privigen, the same manufacturer that was used for treatment (Figure 4B). Serum from a patient obtained 83 days after an GII.4 infection had a virus neutralization titer of > 1280 while preinfection serum was unable to neutralize norovirus replication. Both batches of Ig also neutralized GII.4 norovirus in vitro and had a virus neutralization titer of 640. Amino acid

differences between the antigenic domains of GII.4 Sydney 2012[P31] and the patient strain are shown in Supplementary Table 3.

## DISCUSSION

Here, we evaluated several treatment strategies in a chronically norovirus-infected CVID patient and in vitro. Of these treatments, only treatment with nasoduodenal administered Ig





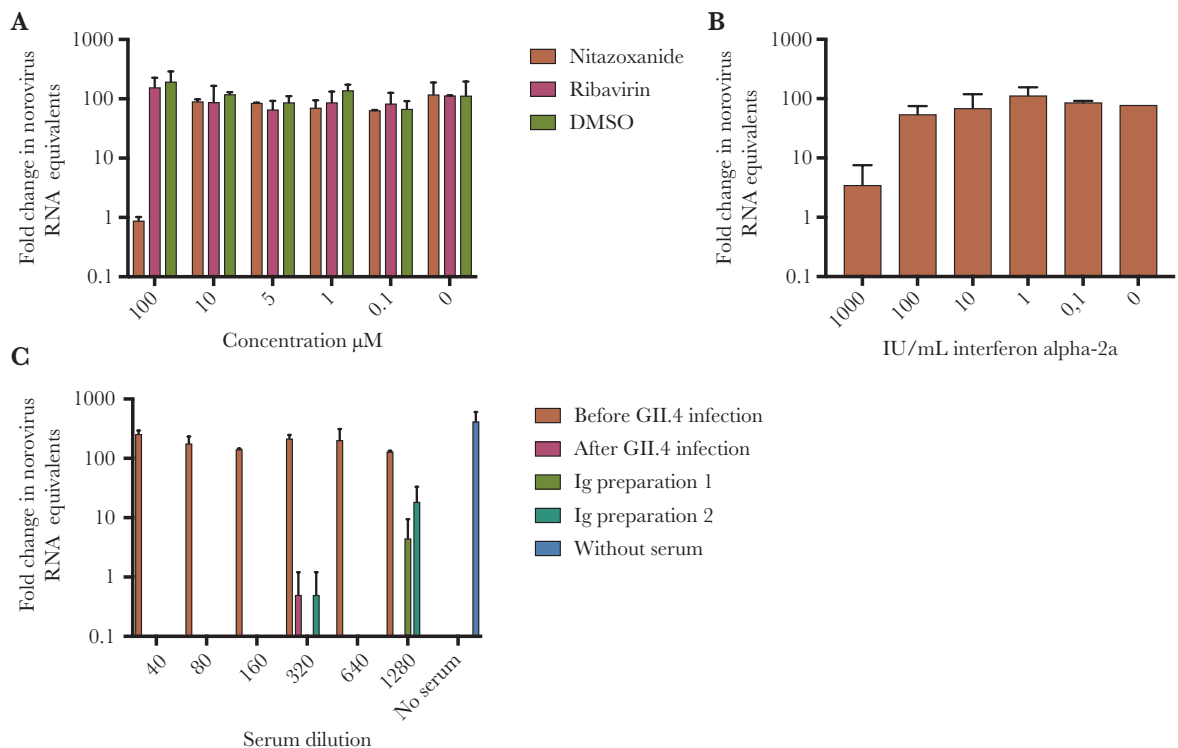
**Figure 3.** Accumulated number of nucleotide (A) and amino acid substitutions (B) in the viral genome compared to day 1 (March 2017), observed in the consensus sequences during treatment. The grey areas represent the duration of treatments. Treatment 1, 14 days of oral nitazoxanide, 500 mg twice daily. Treatment 2, 96 days of oral ribavirin, 400 mg twice daily. Treatment 3, 39 days of interferon alpha-2a, subcutaneous injections of  $3 \times 10^6$  units 3 times per week. Treatment 4, 2 days of oral Ig, 25 mg/kg 4 times daily.

resulted in clearance of the virus and neutralization in enteroid cultures.

Norovirus can replicate in vitro in B cells [21], but despite the detection of vRNA in plasma we were unable to retrieve reliable sequences from plasma and detect viral antigen in peripheral blood B cells by fluorescence-activated cell sorting analysis (data not shown), indicating that these were not a major reservoir for norovirus in this patient. In addition,

vRNA was detected in plasma of the patient during IGRT therapy, but when Ig was administered enterally, the patient cleared norovirus from the plasma. This suggests that norovirus RNAemia originated from spill over from the intestinal tract.

Currently, GII.4 Sydney 2012 is the major variant and replaced the previous variant GII.4 New Orleans 2009 [37]. In chronically infected individuals, noroviruses can evolve to



**Figure 4.** A, Effect of nitazoxanide and ribavirin on norovirus replication in human intestinal enteroid cultures compared to the dimethyl sulfoxide (DMSO) control. At a concentration of 100 μM nitazoxanide all cells were detached 72 hours after mock or virus inoculation. B, Effect of interferon alpha-2a on norovirus replication in human intestinal enteroid cultures. C, Virus neutralization with pre- and post-norovirus-infection serum from a patient with an acute GII.4 infection and 2 immunoglobulin (Ig) batches from Privigen. For each condition norovirus replication is shown as fold change of norovirus RNA equivalents at 72 hours postinoculation compared to 2 hours postinoculation. Error bars denote standard deviation, experiments were performed with two technical replicates.

strains that are genetically divergent to those circulating in the general population [38]. The patient was infected with a GII.4 strain related to a GII.4 New Orleans 2009[P4] strain that, in the phylogenetic tree, was clearly genetically divergent from other GII.4 New Orleans 2009[P4] viruses. The sequences that cluster together on the second longest branch in Figure 2 were from another immunocompromised patient with a chronic norovirus infection [10]. An accumulation of genetic changes was observed over the course of 4 treatment regimens. This was especially evident during ribavirin treatment, and was suggestive of the ribavirin-induced mutagenesis [39].

GII.4 is the most prevalent norovirus genotype and can cause multiple infections during a lifetime, likely resulting in a broad antibody response against GII.4, which will be reflected in the antibody content of Ig preparations from donors. In total 200 mg/kg of Ig was administered over the course of 2 days. For the patient, IgA in peripheral blood was undetectable. It has been estimated that 3 grams of IgA is secreted in the intestinal lumen every day [40], and much lower levels of IgG. However, in this case the IgA was replaced by providing approximately 4 grams of Ig daily enterally. Most immunocompromised individuals have some level of immunity, albeit lower. It is possible that the Ig only helps to temporarily reduce the amount of infectious virus in the intestine, just enough to tip the balance, giving the patient the opportunity to clear the remainder of the infection by themselves, rather than neutralizing all virus present.

There are cases where treatment with oral Ig did not result in viral clearance [18–20]. It is possible that in these cases the virus has evolved within the patient and could no longer be recognized by norovirus-specific neutralizing antibodies in the Ig [38]; another possibility is that the infection was caused by a strain that does not commonly infect adults, resulting in low or nonexistent levels of neutralizing antibodies in the Ig. Ideally, Ig preparations would be standardized for neutralizing norovirus antibody levels or convalescent plasma could be used, as was done for severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) [41].

Only Ig had an effect on virus replication in vivo and in vitro. Interferon alpha-2a had an effect on virus replication in vitro, but at a much higher concentration than reported for the norovirus replicon system [42]. An alternative that was not explored here is combination therapy, which may lead to enhanced antiviral activity. A limitation of this study is that the patient's strain could not be cultured and that another GII.4 strain was used. Ideally, patient-derived strains would be used to evaluate antivirals and Ig prior to treatment of the patient, because there could be strain-specific differences in how effective an antiviral or Ig is. It is not clear why this strain did not replicate in vitro, despite efficient replication in the host. A pediatric cohort study showed that norovirus strains from chronic infections can replicate in vitro, although not all strains and samples showed replication [43].

This study shows that the norovirus culture system can be used to evaluate the effectiveness of antivirals and to evaluate the presence of neutralizing antibodies in Ig preparations. The GII.4 strain from the patient was quite distantly related to all GII.4 strains detected through surveillance in the general population. The fact that the patient could successfully be treated indicates that broad neutralizing antibodies were present in the Ig isolated from healthy donors, at least for GII.4 strains. Finally, a better understanding of the norovirus genotypes and variants detected in chronically infected patients and how these relate to those in the general population will help improve treatment strategies.

#### Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online. Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

#### Notes

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