Genetic study of SARS-CoV-2 nsp12 in non-responder COVID-19 patients to remdesivir

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Review Timeline:

| Event                  | Date       |
|------------------------|------------|
| Submission Date        | June 28, 2022 |
| Editorial Decision     | July 24, 2022 |
| Revision Received      | August 22, 2022 |
| Accepted               | September 2, 2022 |

Editor: Abimbola Kolawole

Reviewer(s): Disclosure of reviewer identity is with reference to reviewer comments included in decision letter(s). The following individuals involved in review of your submission have agreed to reveal their identity: ASAAD MOHAMMED ATAA (Reviewer #1); Jonathan Daniel Hulse (Reviewer #3)

Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

DOI: https://doi.org/10.1128/spectrum.02448-22
July 24, 2022

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Re: Spectrum02448-22 (Genetic study of SARS-CoV-2 nsp12 in non-responder COVID-19 patients to remdesivir)

Dear Dr. Marta Santos Bravo:

Thank you for submitting your manuscript to Microbiology Spectrum. When submitting the revised version of your paper, please provide (1) point-by-point responses to the issues raised by the reviewers as file type "Response to Reviewers," not in your cover letter, and (2) a PDF file that indicates the changes from the original submission (by highlighting or underlining the changes) as file type "Marked Up Manuscript - For Review Only". Please use this link to submit your revised manuscript - we strongly recommend that you submit your paper within the next 60 days or reach out to me. Detailed instructions on submitting your revised paper are below.

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Editor, Microbiology Spectrum

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Reviewer comments:

Reviewer #1 (Comments for the Author):

Dear authors,  
I would like to thank you for your work. Your work is impressive in this section and gives us some important points about Remdesivir as an antiviral SARS-CoV-2. It might give scientists a new way to cure COVID-19.

These my comments:-

Lines (27-28) We need to define genetic and biochemical pathways to RDV resistance and emphasize the need for additional
studies to define the potential for emergence of these or other RDV resistance mutations in clinical settings. So you need to edit this paragraph.

In lines (33 and 36) You need to review numbers

In line (118) Why did not add a gender variable?

In lines (153-160) There is concern about the death rate, which is high compared to the number of patients who received RDV. Are there drug interactions or the effect of this drug on the chronic diseases of patients and old age? This should be clarified.

In lines (215-216) Can you explain this paragraph?

In lines (232-235) You need to add more information to clear up this confusion.

In line (401) Figure 2, you need to change the color of words to make them clear to the audience.

No statistical software was used to analyze the results.

Reviewer #2 (Comments for the Author):

Bravo and co-workers reported a clinical series of some 100+ covid-19 cases with remdesivir treatment and associated viral whole-genome NGS data. They reported that multiple mutations on the nsp12 (RdRp) gene were found but they were not on or in the vicinity of the known RdRp active site and concluded that no virological resistance was found during short (5-day) and longer (5+) courses of remdesivir therapy. Integrated clinical and virological data are important to the field. But this reviewer identified technical flaws in the study design that should be properly addressed before supporting the conclusion of the present work.

Major
1. This study was not a case-control study and there was no functional validation of the mutations in vitro or in cell line to disprove their association with remdesvir resistance. In addition, the authors have no attempts to provide evidence that those sequences collected with remdesivir therapy has mutation rate comparable to background level (i.e., without remdesvir treatment) to support their claims that those mutations are not remdesivir-driven. As the authors performed WGS, such data should be readily available for comparison with those already reported in the literature. This is critically needed before jumping to any conclusions currently based on homologous modelling only without any functional validation.
2. In retrospect, the authors should have collected samples on an ideally daily basis so that those from non-responders could also be sequenced and analysed as long as they remained viral RNA positive and could have served as a comparison group for mutation rate and de novo mutation appearance. The inclusion of another control group comprising COVID-19 patients without remdesivir treatment is also essential. For example, a similar random mutation patterns between treatment and no-treatment groups can indirectly indicate that the mutations were not associated with antiviral resistance. The key message is that proving and disproving association of mutations to antiviral resistance require the same par of evidence that the current study unfortunately lack.

Minor
3. Line 133: "retrotranscribed" should have read as "reverse-transcribed".
4. Lines 133-136: More technical descriptions on the NGS workflow would be very helpful. Simply saying "as previously described" is far from reader-friendly. At least the authors should provide name of the pipeline and key algorithm used in the analysis NGS data.
5. Bioinformatics pipeline and cut-off/ threshold used in the identification of purported mutations associated (or not associated) with remdesivir therapy needs to be clearly described. For example, sequences existed as quasispecies and did the mutations need to be present in 100% of the illumine reads in order to qualify as a mutation? If no, what was the selection criteria and the rationale behind?
6. Figure 1: the "Positive sgRNA" and "Negative sgRNA" labels should indicate that they referred to samples collected at the last day of remdesivir dosing.
7. Lines 157-160: What do the numbers in parenthesis refer to, IQR or range or something else? Please clarify.
8. The work would benefit from English-editing.

Reviewer #3 (Comments for the Author):

This is a very interesting paper and I feel that it adds to the body of work surrounding COVID-19. Here are some suggestions:
Line 27: Delete 'of', it should read "No evidence of global widespread 28 RDV-resistance mutations has been reported"

Line 28 - 30: Change wording from 'or' into 'to'. "Determining emergent mutations prior to..."

Line 32: Why is there a (63.2%) in the sentence. Needs clarification.

Line 33 -34: Clarify Next Generation Sequencing. 454 Pyrosequencing or Illumina, or some other type?

Line 40 - 42: Try not to use 'and' two times in one sentence. Substitute one of the 'and' for 'as well as'

Line 47: Capitalize Remdesivir or use RDV since it was defined earlier.

Line 49: Capitalize Remdesivir or use RDV since it was defined earlier.

Line 79: Capitalize the drug names. They are proper nouns.

Line 89: Add a comma after in vitro

Line 90: Add a comma after V557L

Line 116: Clarify Next Generation Sequencing. 454 Pyrosequencing, Illumina, or some other type?

Line 157: Clarify what (54;73) means. Is this 54 - 73 years old?

Line 178: Remove the second 'only' in the sentence. It should read, "The only non-synonym mutation detected..."

Line 181: Try to avoid using 'and' two times in one sentence. Change the 'and' to 'as well as'

Line 194: Be constant with your italics of nsp12.

Line 196: Be constant with your italics of nsp12.

Line 195-198: Run-on sentence. Break into two sentences if possible.

Line 207: Be constant with your italics of nsp12.

Line 209: Be constant with your italics of nsp12.

Line 213 - 217: Avoid using 'and' multiple times in a sentence. Break this sentence into 1-3 sentences because it is to long.

Line 410: Make sure that in Table 1, all of your Clinical Characters are Capitalized. Be consistent.

Staff Comments:

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Thank you for submitting your paper to Microbiology Spectrum.
Genetic study of SARS-CoV-2 nsp12 in non-responder COVID-19 patients to remdesivir

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ABSTRACT

Remdesivir (RDV) was the first antiviral drug approved by the FDA to treat severe COVID-19 patients. RDV inhibits SARS-CoV-2 replication by stalling the non-structural protein 12 (nsp12) subunit of the RNA-dependent RNA polymerase (RdRp). No evidence of global widespread of RDV-resistance mutations has been reported. Determining emergent mutations prior or subsequent antiviral therapy has strong implications for clinical management and virus surveillance.

This study identified 57/149 (38.3%) patients who did not respond to one course (5-days) (63.2%) or prolonged (5-20 days) (36.2%) RDV therapy by subgenomic RNA detection. Genetic variants in the nsp12 gene were detected in 17/49 (34.7%) non-responder patients by next-generation sequencing, including the de novo E83D mutation that emerged in an immunosuppressed patient after receiving 10+8 days of RDV, and the L838I detected at baseline and/or after prolonged RDV treatment in 8/49 (16.3%) non-responder subjects. Although 3D protein modelling predicted no-interference with RDV, the amino acid substitutions detected in the nsp12 involved changes on the electrostatic outer surface and in secondary structures that may alter antiviral response.

It is important for health surveillance to study potential mutations associated to drug resistance and the benefit of RDV retreatment, especially in immunosuppressed patients and in those with persistent replication.

Importance

This study provides clinical and microbiologic data of an extended population of hospitalized patients for COVID-19 pneumonia who experienced treatment failure, detected by the presence of subgenomic RNA. The genetic variants found in the nsp12 pharmacological target of remdesivir bring into focus the importance of monitoring emergent mutations, one of the objectives of the World Health Organization (WHO) for health surveillance. These mutations...
become even more crucial as remdesivir keeps being prescribed and new molecules are being repurposed for the treatment of COVID-19.

The present article offers new perspectives for the clinical management of non-responder patients treated and retreated with RDV, and emphasizes the need of further research of the benefit of combinatorial therapies and RDV retreatment, especially in immunosuppressed patients with persistent replication after therapy.

**Keywords:** COVID-19, remdesivir, resistance mutations, subgenomic RNA, retreatment

**Word count:** 200 abstract; 2492 full-text
The global pandemic of novel coronavirus disease 2019 (COVID-19) caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has created an urgent effort to repurpose antiviral inhibitors to control viral replication and improve clinical outcomes [1]. Remdesivir (RDV) was originally developed in response to the 2014-2016 Ebola outbreak in West Africa [2] and has shown broad-spectrum activity in vitro and in vivo against pathogenic human coronaviruses, including the novel SARS-CoV-2 [3]. RDV was the first drug to be approved by the FDA in October 2020 and has been extensively used in clinical practice during the COVID-19 pandemic in hospitalized patients [4]. Recently, two oral prodrugs have also been approved for treatment of COVID-19 patients: molnupiravir and nirmatrelvir/ritonavir [5, 6].

RDV, formerly GS-5734, is a nucleoside analogue pro-drug that inhibits the non-structural protein 12 (nsp12) subunit of the RNA-dependent RNA polymerase (RdRp) by competing with its usual natural substrate adenosine triphosphate [7]. The nucleoside analog is incorporated into the generating RNA strand and evade proofreading to successfully inhibit viral RNA synthesis. Several clinical trials and a recent control-case study demonstrated reduced time to recovery, hospitalization time, morbidity and mortality [8-10].

One of the major concerns for health surveillance is determining emergent mutations that could be associated to drug resistance, fitness advantage, immune escape, or better adaptation to the host. Only few studies have attempted to characterized amino acid substitutions that could confer resistance to RDV by in silico prediction, in vitro or in animal models [7, 11-15]. For instance, F480L, V557L and E802D has been described to conferred 2.4x, 5.2x, 6x-fold decrease sensitivity to RDV in vitro, respectively [7, 14]. Moreover, E802D was recently detected in an immunocompromised patient after RDV therapy [15], however, no other resistant clinical case or evidence of global widespread transmission of RDV-resistant mutants after treating with RDV for over a year have been described thus far.
This study aimed to identify novel genetic variations in the nsp12 gene in clinical samples before and after RDV therapy in severe COVID-19 patients who did not respond to therapy with RDV.

**MATERIALS**

**Study population**

This was an observational prospective study that included 149 COVID-19 patients admitted in the Hospital Clinic of Barcelona (Spain), from February 2021 until November 2021, who filled the criteria to receive RDV according to the recommendations of the Spanish Medicine Agency. These criteria were the following: (1) SARS-CoV-2 positivity confirmed by RT-PCR, (2) ≤7 days from symptoms onset, (3) radiological signs of pneumonia, (4) requiring supplemental oxygen support or respiratory rate ≥24 breaths per minute or PaO₂/FiO₂ <300 mmHg. The RDV dose used was 200 mg as a loading dose the first day and 100 mg/24h for the next 4 consecutive days. Some immunosuppressed patients received prolonged remdesivir therapy (>5d-RDV), decision made by the physician in charge according to the clinical evolution and the immune status of the patient.

Nasopharyngeal/throat swabs were collected before 1st RDV dose and after the last dose for each patient. Both samples were tested for SARS-CoV-2 genomic (gRNA) and subgenomic RNA (sgRNA) by reverse transcriptase-real time polymerase chain reaction (RT-PCR). Although there is some controversy concerning the use of sgRNA to detect active viral replication, we previously validated this technique with viral culture [16]. Non-responder subjects were considered when sgRNA was detected at the end of the RDV treatment. Nucleotide changes were determined by next generation sequencing (NGS) in pre-and post-RDV treatment clinical samples of non-responders. Novel amino acid substitutions found in clinical isolates were evaluated in 3D modelling structure of the protein in silico. This study design is showed in figure 1.
Clinical data were collected to study the overall population, considering the following variables: age, significant comorbidities, days of treatment with RDV, Intensive Care Unit (ICU) admission, and all-cause mortality.

**RT-PCR for genomic and subgenomic RNA detection for SARS-CoV-2**

The presence of SARS-CoV-2 gRNA was determined by real-time RT-PCR in the automatic system Cobas 6800 (Roche, Barcelona) according to the manufacturer’s instructions. Inactivation was performed using 1:1 volume of Cobas Omni Lys (Roche, Germany) and total nucleic acid extraction was done using MagNA Pure Compact (Roche, Switzerland). Elutes were used for sgRNA test and NGS. *Envelope (E)* sgRNA was detected by real-time RT-PCR following the procedure previously described [16]. Throat/nasopharyngeal swabs and elutes were aliquoted and stored at -80°C since their testing. Cycle threshold (Ct) values >40 for gRNA or sgRNA RT-PCRs were considered negative.

**Next Generation Sequencing of SARS-CoV-2 complete genome**

Retrospectively, the eluted RNA of the SARS-CoV-2 were retrotranscribed into cDNA and SARS-CoV-2 complete genome amplification was conducted following the openly available protocol developed by the ARTIC network [17] using Illumina platform. The sequences obtained went through a bioinformatic pipeline based on a previously described open-source pipeline [18]. Mutations were identified by aligning the consensus sequence with the Wuhan-Hu-1 reference genome [GenBank: MN908947.3] using Nextclade v.1.14.0. Quality of the sequences were determined by the quality control metrics of this tool.

**Lineage identity**

The lineage of SARS-CoV-2 was identified using Nextclade v.1.14.0 according to the amino acid replacements determinant of each variant as classified by the SARS-CoV-2 Interagency Group (SIG) and the Centres of Diseases Control and Infection (CDC) [19].
Molecular modelling of mutations in the SARS-CoV-2 nsp12 and spike proteins

Mutations found genotypically in the nsp12 subunit of the RdRp were generated in the ribbon structure of the cryo-EM model of the replicating SARS-CoV-2 polymerase complex (PDB 6YY7) using PyMOL Molecular Graphics System (Schrödinger, version 2.5.2). This software was also used for structural visualization. Adaptive Poisson-Boltzmann Solver (APBS) Tool 2.1 Electrostatic Plugin included in PyMOL was used for macromolecular electrostatics calculations and to display the results as an electrostatic potential molecular surface.

Ethical approval

The Ethical Committee of our institution accepted the protocol (HCB/2021/0080) and the included patients signed the informed consent to participate in the study.

RESULTS

In a cohort of 149 patients hospitalized for COVID-19 pneumonia, 111 received a 5-dose course of RDV (5d-RDV) and 38 received prolonged RDV therapy (>5d-RDV) (figure 1). The median age of the 5d-RDV subset was 62.7 (54; 73) years-old, 81 (73%) presented at least one comorbidity, 34 (30.6%) were admitted in the ICU and 7 (6.3%) died by all-cause mortality (table 1). The median age in the >5d-RDV subgroup was 59 (53; 67) years-old and they were treated with RDV for a median of 10 (7; 21) days. Of the 38 patients, 33 (86.8%) had at least one comorbidity, 19 (50%) were admitted in the ICU and 8 (21.1%) died by all-cause mortality, but 3 deaths were not related to COVID-19. Extended clinical data of both subsets is shown in table 1.

SARS-CoV-2 sgRNA identified 57/149 (38.3%) patients with actively replicating virus after treatment, classified as non-responders (figure 1). There were 36/57 (63.2%) non-responders in the 5d-RDV subgroup and 21/57 (36.8%) from the >5d-RDV subgroup. The 17 remaining patients of the >5d-RDV cohort were not tested for sgRNA and were discarded because the
pre- or post-treatment swab could not be recovered. Ct values corresponding to the gRNA RT-PCR were maintained or decreased during treatment in non-responder patients. Nucleotide changes in the nsp12 gene were detected by NGS and were compared before and after RDV therapy in non-responder patients. Sequencing quality was achieved in both samples in 28/34 patients of the 5d-RDV subset, and in all 21 patients of the >5d-RDV subset.

NGS allowed the detection of 18 nsp12 nucleotide substitutions detected in 17/28 (60.7%) patients from the 5d-RDV subgroup and in 12/21 (57.1%) patients receiving prolonged RDV therapy. Of the 18 nucleotide substitutions identified, 9 conferred an amino acid substitution, pointing genetic evolution after treatment (table 2). Mutations were either detected at baseline (pre-RDV), before and after treatment (pre/post-RDV) or emerged de novo after treatment (post-RDV). The only non-synonym mutation only detected after treatment was E83D. E83D emerged in a patient (patient 7, table S1) with diffuse large B-cell lymphoma that was treated with R-CHOP combination chemotherapy and with several antiviral drugs (lopinavir, ritonavir, hydroxychloroquine, azithromycin, RDV) and convalescent plasma. E83D mutation emerged de novo after 2 courses (10 + 8 days) of RDV. At the end, this patient was admitted in the ICU and died after 8 months of SARS-CoV-2 infection. The most frequent mutation (18.4%) was L838I, found at baseline in 1 patient and after prolonged treatment in 8 non-responder subjects. Clinical and genetic data of all patients with de novo mutations is shown in table S1.

NGS allowed the determination of SARS-CoV-2 lineages. Alpha (B.1.1.7) variant was mainly detected in subjects with 5d-RDV (n=19) compared to >5d-RDV ones (n=3), whereas delta (B.1.167) was more predominant in >5d-RDV subjects (n=12) than in the 5d-RDV subgroup (n=4). The variant B.1.177 emerged as an outbreak in Spain during the summer of 2020, and its incidence was similar in both groups (5d-RDV n=5; >5d-RDV n=6).

Predicting phenotype of novel nsp12 mutations by 3D protein modelling
The position of all genetic variants detected at any time of the treatment were located in the gene structure of the nsp12 (figure 2) and non-synonym mutations in a 3D protein structure of the RdRp (figure 3A). *In silico* studies postulated that RDV binds strongly to the active pocket of the nsp12 by electrostatic interaction with residues R553, R555, T556, K551, W617, D618, Y619, D623, S682, N691, D760, D761, A762, W800, E811, F812, K813, and S814, and by Van der Waals bonds in the K621, K622, D623, and L758 [11, 12]. Our genotypic results showed the emergence of mutations located next to residues involved in RDV-nsp12 binding (E729D, D738Y, L838I), in the nsp8-nsp12 interaction region (P227L), and inside the RNA-binding domain of the polymerase (T422I). However, RdRp 3D protein modelling shows that all novel amino acid substitutions are distant from the RNA binding domain, where the RDV inhibits the polymerase activity. Replacing one amino acid of a helix for another can change repartition of amino acids exposed to solvent, as found for the E729D (figure 3B). Slight changes in conformation were detected for the remaining amino acid substitution.

Most mutations changed a negative charged amino acid to aliphatic-chained ones, substituting the highly negative electrostatic outer surface of the nsp12 to a major overall neutral status of the surface (figure 3C). Changes in polarity, charge, and size of the amino acids could potentially modify interactions between nsp12 and antivirals.

**DISCUSSION**

The study of sgRNA allowed the identification of 57/149 (38.3%) patients who did not respond to 5-days or >5-days of RDV therapy. Eighteen genetic variants in the *nsp12* gene were frequently detected in 17/49 (34.7%) by NGS in non-responder subset. No significant viral mutations were determined to be associated to the failure to RDV treatment, except for the *de novo* E83D mutation that emerged after receiving 18 days of RDV, and the L838I mutation, which was found at baseline in 1 patient and after prolonged treatment in 8 (18.4%) non-responders, and its localized next the previously E802D RDV-resistant mutation.
Genetic variants in nsp12 arose similarly after 5 days of RDV or longer treatment independently of the duration of therapy (5d-RDV 57.1% vs. >5d-RDV 60.7%) and, surprisingly, 5d-RDV subjects presented more de novo mutations (n=6) than >5d-RDV (n=3). Almost all mutations were detected at baseline in at least one subject showing an evolutionary tuning of the viral proteins to a new host, although a response for antiviral selective pressure cannot be excluded in those that persist after treatment. In non-responder patients, viral loads progressively increased during treatment, as Ct values were maintained/decreased and sgRNA remained detectable, which could be due to either slow viral shedding or failure to treatment. Despite the high frequency of substitutions in nsp12, none of them have been previously described to confer resistance to RDV [15]. We detected the L83I mutant, nearby the E802D RDV-resistant mutation, in 8 patients before and after treatment, and in 1 patient only post-therapy. All of them received either 10, 20 or 21 days of RDV therapy, 6 of them were admitted in the ICU and 3 died, thus, it seems to be associated to a worse prognosis, although the number of cases is scarce to confirm it. It suggests that this naturally occurring variant may provide an improved viral escape of the inhibitor, since no evidence that RDV acts as a mutagen driving spontaneous mutations has been reported and the template stalling action of the RDV limits spontaneous mutations emergence [7, 20].

E83D emerged in the SARS-CoV-2 delta variant that infected an immunosuppressed patient, who was admitted in the ICU and died after 9 months of infection. The E83D mutation was detected after RDV retreatment (10 + 8 days), but not before, making it potential for phenotyping. 3D protein modelling did not predict any interference in the interaction with RDV, however, its implication in fitness advantage, inhibitor escape or adaption to the host is unknown.

E729D and D738Y may also have an implication of antiviral response, as they were located in the palm subunit of the polymerase active site next to the residues involve in the RDV-nsp12
interaction and E729D also modified an alpha-helix structure. T422I is located in the conserve motif G in charge of the RNA template attachment. It is close to the previously described resistant mutations in vitro (F480L, V557L) and in vivo in an immunocompromised patient with persistent viremia (D484Y) [7, 21]. They did not alter the RdRp catalytic site but are thought to impact RdRp fidelity checking step before catalysis. Molecular surveillance of this region in RDV-treated COVID-19 patients is suggested to be warranted [20]. Even though 3D protein modelling predicted none of the mutations found in this study block the binding pocket of RDV, they involved changes on the electrostatic outer surface and in secondary structures that may alter antiviral response.

Lineages could be a concern of the severity of the disease and the antiviral response. Alpha variant was more frequent in the 5d-RDV and delta in the >5d-RDV, which agrees with its higher pathogenicity. Besides the possible bias caused by the time of inclusion of the patients, delta lineage could influence on a worse response to RDV and the need of a second course of treatment.

Available literature about RDV retreatment only reported few clinical cases [22-23] but no large studies have been carried out, making this practice still unaddressed in current treatment guidelines [24]. However, this study provides further information of the response to RDV treatment and retreatment in patients whose active replication was previously checked by sgRNA and clinical data was exposed.

In conclusion, no significant virological resistance was determined after different courses of RDV in non-responders severe COVID-19 patients and the duration of RDV treatment does not seem to be a risk factor for developing RDV-resistance mutations. However, mutations found in this study, especially E83D, were potential to be further evaluated by recombinant phenotyping. It is crucial to monitor antiviral resistance as one of the objectives of the World Health Organization (WHO) for health surveillance, and to study the potential benefit of
combinatorial therapies and RDV retreatment, especially in immunosuppressed patients or with persistent replication.

**Funding**

This work was financed by a Gilead Sciences grant (IN-ES-540-6089). This work was financed by ad hoc patronage funds for research on COVID-19 from donations from citizens and organizations to the Hospital Clínic de Barcelona-Fundació Clínic per a la Recerca Biomèdica.

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FIGURES

Figure 1. Scheme of the study design. Patients positive for SARS-CoV-2 that were admitted in the Hospital Clinic of Barcelona (Spain) for COVID-19 pneumonia and were treated with remdesivir (RDV) were included in the study. They were treated with 5 doses (1 course) of RDV or with longer treatments (>5 doses). Subgenomic RNA (sgRNA) detection was performed in all samples in order to detect viral replication before and after treatment. Patients with positive sgRNA after treatment were classified as non-responders and were sequenced by next-generation sequencing (NGS). Not all clinical samples could be sequenced with enough quality to be analysed and included in the study due to RNA degradation or low viral load in the sample. Of the samples from the >5-dose subpopulation that could be studied, all were sgRNA RT-PCR positive in the last sample. N indicates the number of subjects included in each cohort.
Figure 2. Gene structure of the SARS-CoV-2 non-structural protein 12 with the novel mutations detected. The nucleotide position is indicated respect to Wuhan-Hu-1 reference genome [GenBank: MN908947.3]. Amino acid substitutions are indicated in brackets. Gene structure is based on Gao et al, 2020 [25].
Figure 3. Location of novel RdRp mutations in 3D protein models. (A) Theoretical structure of RdRp is represented in the ribbon structure of the cryo-EM model using PyMOL. The different subunits of the RdRp are colored as follow: nsp7 in blue, nsp8a in yellow, nsp8b in pink, nsp12 in green. The RNA duplex is colored in orange and blue. Novel genotypically detected mutations indicated in red, in the right figure. (B) E729D mutation breaks the alpha-helix secondary structure of the nsp12 (C) APBS-generated electrostatic surface of the RdRp. Negative charged areas are indicated in red and positive charged areas in blue. Mutations are visualized in red and tagged in the figure.
Table 1. Clinical characteristics of the study population according to the treatment of remdesivir received.

| Clinical characteristics                                      | 5d RDV | >5d RDV |
|---------------------------------------------------------------|--------|---------|
| N                                                             | 111    | 38      |
| Age (median; IQR)                                             | 62.7 (54; 73) | 59 (56; 67) |
| Days of RDV therapy (median; IQR)                             | 5      | 10 (7; 21) |
| Comorbidities (n,%)                                           | 81 (73%) | 33 (86.8%) |
| • Hypertension (n,%)                                          | 51 (45.9%) | 8 (21.1%) |
| • Diabetes mellitus (n,%)                                     | 26 (23.4%) | 5 (13.2%) |
| • Obesity (n,%)                                               | 18 (16.2%) | 3 (7.9%) |
| • Cardiovascular disease (n,%)                                | 32 (28.8%) | 3 (7.9%) |
| • chronic pulmonary disease\(^a\) (n,%)                       | 24 (21.6%) | 6 (15.8%) |
| • chronic kidney failure (n,%)                                | 9 (8.1%) | 1 (2.6%) |
| • haematological malignancies\(^b\) (n,%)                     | 15 (13.5%) | 25 (65.8%) |
| • Solid malignancy with active chemotherapy (n,%)             | 4 (3.6%) | 1 (2.6%) |
| • Transplant recipients (n,%)                                  | 4 (3.6%) | 6 (15.8%) |
| • Other disorders treated with immunosuppressors (n,%)        | 7 (6.3%) | 1 (2.6%) |
| ICU admission (n,%)                                           | 34 (30.6%) | 21 (55.3%) |
| Mortality (n,%)                                               | 7 (6.3%) | 8 (21.1%) |

\(^a\) Chronic pulmonary disease includes chronic obstructive pulmonary disease and asthma

\(^b\) Haematological malignancies include lymphoma or leukaemia

**Abbreviations:** RDV remdesivir, ICU Intensive Care Unit

*Age and days of treatment are indicated as the median and the interquartile range Q1; Q3.*
**Table 2.** *Nsp12* nucleotide substitutions detected at baseline (pre-RDV), at baseline and after treatment with remdesivir (pre/post-RDV) and after (post-RDV) therapy in clinical isolates.

| Mutations\(^a\) | Location | Pre-RDV\(^b\) | Pre/post-RDV\(^b\) | Post-RDV\(^b\) | ICU\(^b\) | Mortality\(^b\) |
|----------------|----------|---------------|----------------------|-----------------|-----------|-----------------|
| A13535G (Y32C) |          | 2             | 1 (10d)              | 1 (5d)          | 1         | 0               |
| C13551T        |          |               | 1 (18d)              |                 | 1         | 1               |
| G13564T (V42L) |          |               | 1 (5d)               | 0               | 0         |                 |
| A13689T (E83D) |          |               | 1 (18d)              |                 | 1         | 1               |
| A13711G        |          |               | 1 (5d)               |                 | 0         | 0               |
| (K91G)         |          |               |                      |                 |           |                 |
| C14119T        | Nsp8-interaction | 1 (5d) | 0                    | 0               |           |                 |
| C14120T        | Nsp8-interaction | 3 (5d n=2, 10d)| 1 (5d) | 3         | 1         |                 |
| (P227L)        |          |               |                      |                 |           |                 |
| C14178T        | Nsp8-interaction | 1 (5d) | 0                    | 0               |           |                 |
| C14547A        | Nsp7-8-interaction | 2 (5d)| 3 (5d)               | 3               | 0         |                 |
| C14703T (T422I)| RNA binding site (motif G) | 1 (10d) |                   | 1               | 0         |                 |
| C15237T        | RNA binding site (motif B) | 1 (10d) |                   |                 | 0         | 0               |
| C15240T        | RNA binding site (motif B) | 1 (5d) |                   | 1               | 1         |                 |
| C15324T        | RNA binding site | 2 (30d) | 2 (5d)               | 3               | 1         |                 |
| C15441T        | RNA binding site (motif C) | 1 (5d) |                   | 0               | 0         |                 |
| G15627T        | RNA binding site (motif E) | 1 (5d) |                   | 1               | 0         |                 |
| (E729D)        |          |               |                      |                 |           |                 |
| C15652T        | RNA binding site | 1 (5d) | 1 (5d)               | 1               | 0         |                 |
| (D738Y)        |          |               |                      |                 |           |                 |
| G15910T        |          |               | 1 (10d)              |                 | 0         | 0               |
| C15952A (L838I)|          | 8 (10d n=7; 21d)| 1 (20d)              | 6               | 3         |                 |

\(^a\) The nucleotide position is indicated respect to Wuhan-Hu-1 reference genome [GenBank: MN908947.3]. Amino acid substitution is indicated in brackets.
Indicate the number of subjects with the specific substitution, admitted in the ICU or died due to all-cause mortality. Days of treatment received when the mutation was found are indicated in brackets. Abbreviations: Nsp non-structural protein, RDV remdesivir, ICU intensive care unit.
**SUPPLEMENTARY DATA**

**Table S1. Clinical characteristics of the patients with SARS-CoV-2 non-structural protein 12 and/or Spike mutations of interest.**

| ID | Age | Comorbidity | ICU admission | Mortality | Immunossuppressive treatment | Antiviral treatment | Date of infection | Linage | NSP12 |
|----|-----|-------------|---------------|-----------|-------------------------------|--------------------|------------------|--------|-------|
| 1  | 60  | Hypertension, enolic dilated cardiomyopathy | no | no | BNB | RDV 5d | March 2021 | Alpha (B.1.1.7) | A13535G (Y32C), C14120T (P227L), C15324T |
| 2  | 68  | Hypertension | yes | no | TCZ, CORT | RDV 5d | April 2021 | Alpha (B.1.1.7) | C14120T (P227L), C15324T |
| 3  | 40  | No | no | no | BNB | RDV 5d | March 2021 | Alpha (B.1.1.7) | G14547A |
| 4  | 47  | No | no | no | TCZ, BNB, CORT | RDV 5d | April 2021 | Alpha (B.1.1.7) | G14547A |
| 5  | 40  | No | yes | no | TCZ, BNB, CORT | RDV 5d | April 2021 | Delta (B.1.167) | G14547A |
| 6  | 49  | Thalassemia minor | no | no | BNB | RDV 5d | June 2021 | Alpha (B.1.1.7) | G15652T (D738Y) |
| 7  | 83  | Diffuse large B-cell lymphoma | yes | yes | R-CHOPa | LPV + RTV + HCQ 7d, AZM 5d, RDV 10d + 8d, plasma | March – December 2021 | Delta (B.1.167) | - |
| 8  | 67  | Kidney transplant, arterial hypertension, hypercholesterolemia | no | no | BNB 10d | RDV 10d | July 2021 | Delta (B.1.167) | G15910T |
| 9  | 64  | Mantle lymphoma in complete remission | yes | No | DEX 10d, TCZ, BNB, anakinra | RDV 20d, TEC, IVM + plasma | August 2021 | Delta (B.1.167) | C15952A (L83I) |

*a R-CHOP is a chemotherapy composed by the combination of rituximab, cyclophosphamide, hydroxidaurubicine, oncovin and prednisone

**Abbreviations:** ICU intensive care unit, LPV lopinavir, RTV ritonavir, RDV remdesivir, HCQ hydroxychloroquine, AZM Azithromycin, TEC teicoplanin, IVM ivermectin, TCZ tocilizumab, BNB baricitinib, DEX dexamethasone, CTX cyclophosphamide, PDN prednisone, CORT other corticoids.
Response to reviewers

Reviewer comments:

Reviewer #1 (Comments for the Author):

Dear authors, I would like to thank you for your work. Your work is impressive in this section and gives us some important points about Remdesivir as an antiviral SARS-CoV-2. It might give scientists a new way to cure COVID-19.

These my comments:

Lines (27-28) We need to define genetic and biochemical pathways to RDV resistance and emphasize the need for additional studies to define the potential for emergence of these or other RDV resistance mutations in clinical settings. So you need to edit this paragraph.

We rewrote it with your comments, however, the paragraph indicates the objective of the study that consists on studying genetic variants before and after remdesivir therapy in clinical samples.

In lines (33 and 36) You need to review numbers

Thank you very much for your correction. We change the denominators and the percentages of each subgroup of non-responder patients.

In line (118) Why did not add a gender variable?

Thank you for your comment. We added the gender of both subgroups in table 1 and in the manuscript in lines 156, 157 and 161.

In lines (153-160) There is concern about the death rate, which is high compared to the number of patients who received RDV. Are there drug interactions or the effect of this drug on the chronic diseases of patients and old age? This should be clarified.

The mortality rate of the 5d-RDV subgroup is 6.3% which is not high considering all of them were hospitalized patients for COVID-19 pneumonia and 30% were admitted in ICU. Similarly, the approximately 30% of the >5-d RDV subgroup is not high considering 55% were admitted in ICU. The clinical history of the last subgroup indicates that >85% had comorbidities that are associated with worse outcomes and the majority received immunosuppressive therapy.

There is a warning about possible drug interactions between immunosuppressive drugs and those already approved or under investigation for the treatment of COVID-19. Currently, there is no data on possible interactions between RDV and immunosuppressive drugs, unlike chloroquine/hydroxychloroquine and lopinavir/ritonavir (Li et al, 2020).

Li Y, Yang N, Li X, Wang J, Yan T. Strategies for prevention and control of the 2019 novel coronavirus disease in the department of kidney transplantation. Transplant International. 2020;33(9).

In lines (215-216) Can you explain this paragraph?

Genetic variants were studied before and after treatment in patients receiving RDV for 5 days and in patients receiving RDV from 6 to 20 days. It was expected that genetic variants emerged more frequently in patients with longer treatments as they had more chance to emerge and be selected by the drug, however, our results showed that mutations were detected equally in both
groups, suggesting it does not depend on the duration of the therapy. This information has been clarified in lines 220-221.

In lines (232-235) You need to add more information to clear up this confusion.

We described the clinical case of the patient where the variant E83D was detected. As this mutation was only detected after RDV therapy, we phenotyped it by 3D protein modelling and its location suggested there is no interference with the mechanisms of action of RDV, but there are multiple implications that the mutation might cause that were not confirm, such as an altered replicative capacity or a better adaption to the host by evading the immune system.

In line (401) Figure 2, you need to change the color of words to make them clear to the audience.

Thank you for your comment. I think you meant figure 3 as it is the one corresponding to line 401. We tried to interfere the minimum possible on the figure so the 3D protein structure can be correctly visualized and the localization of the amino acid. However, we will change the background of each tag to black with white font.

No statistical software was used to analyze the results.

There is not statistical analysis in the study that need to be performed. The maximum degree of mathematics data presented are the N (number of subjects or mutations) and the percentage in relation to the total of samples of the group. We used clinical data to describe the clinical history in which the genetic variants were detected, but not to determine factors associated with COVID-19 diseases, that is the reason why we did not show significance either associations.

Reviewer #2 (Comments for the Author):

Bravo and co-workers reported a clinical series of some 100+ covid-19 cases with remdesivir treatment and associated viral whole-genome NGS data. They reported that multiple mutations on the nsp12 (RdRp) gene were found but they were not on or in the vicinity of the known RdRp active site and concluded that no virological resistance was found during short (5-day) and longer (5+) courses of remdesivir therapy. Integrated clinical and virological data are important to the field. But this reviewer identified technical flaws in the study design that should be properly addressed before supporting the conclusion of the present work.

Major

1. This study was not a case-control study and there was no functional validation of the mutations in vitro or in cell line to disprove their association with remdesivir resistance. In addition, the authors have no attempts to provide evidence that those sequences collected with remdesivir therapy has mutation rate comparable to background level (i.e., without remdesivir treatment) to support their claims that those mutations are not remdesivir-driven. As the authors performed WGS, such data should be readily available for comparison with those already reported in the literature. This is critically needed before jumping to any conclusions currently based on homologous modelling only without any functional validation.

This is an uncontrolled before-and-after study or intervention study in which we evaluated the genetic variants before and after treatment in each individual subject, therefore, every mutation is compared with the background of the sample collected previously to remdesivir therapy. We did not compare it with a control subpopulation that has never received remdesivir because
those sequence are publicly available in many repositories. Our results suggested that mutations that only emerged after treatment but were not at baseline in the same subject, and were not reported to be specific of a new lineage, could be driven by remdesivir therapy. Effectively, the results of the WGS are available and we checked that those variants were not reported previously elsewhere.

2. In retrospect, the authors should have collected samples on an ideally daily basis so that those from non-responders could also be sequenced and analysed as long as they remained viral RNA positive and could have served as a comparison group for mutation rate and de novo mutation appearance. The inclusion of another control group comprising COVID-19 patients without remdesivir treatment is also essential. For example, a similar random mutation patterns between treatment and no-treatment groups can indirectly indicate that the mutations were not associated with antiviral resistance. The key message is that proving and disproving association of mutations to antiviral resistance require the same par of evidence that the current study unfortunately lack.

In the design of the study, we firstly thought to include a control group of subsets infected with SARS-CoV-2 that were not treated with remdesivir. However, in our hospital, it was impracticable to have patients without remdesivir treatment, since those who met the criteria received remdesivir since the beginning of the pandemic. Moreover, variants were evolving very fast and each virus had different baseline mutations that needed to be considered in order to appropriately study the selection by the antiviral drug. Therefore, we concluded that the best control we could add is to have a sample from the same patient collected before receiving remdesivir, so the sequencing result could have the same clinical background in every case, with the only study variable of remdesivir therapy.

Minor

3. Line 133: "retrotranscribed" should have read as "reverse-transcribed".

Corrected.

4. Lines 133-136: More technical descriptions on the NGS workflow would be very helpful. Simply saying "as previously described" is far from reader-friendly. At least the authors should provide name of the pipeline and key algorithm used in the analysis NGS data.

Thank you for your comment. The name of the pipeline has been added in the method section (line 136). This is a public pipeline which is available at the following link:

https://gitlab.com/fisabio-ngs/sars-cov2-mapping/

The workflow for Illumina data is based on iVar and consists of the following steps:

1. Quality trimming
2. Mapping of reads to MN908947.3 reference genome
3. Primer trimming
4. Consensus genome generation
5. Variant calling
6. Basic QC summaries

I think your comment is very helpful, however, we consider this information very technical to be added in the methods section and it could be easily followed in the website referenced in the manuscript.
5. Bioinformatics pipeline and cut-off/ threshold used in the identification of purported mutations associated (or not associated) with remdesivir therapy needs to be clearly described. For example, sequences existed as quasispecies and did the mutations need to be present in 100% of the illumine reads in order to qualify as a mutation? If no, what was the selection criteria and the rationale behind?

The variant was considered in the consensus sequence generated after the bioinformatic analysis when it was present in at least 80% of reads with a minimum quality of 20 and minimum depth per position of 30:

MINQUAL_CONS=20     # Minimum quality for consensus calling
MINFREQ_CONS=0.8     # Minimum frequency to consider fixed a SNP in consensus
MINDEPTH_CONS=30     # Minimum position depth, ambiguous_char otherwise
AMBIGUOUS_CHAR=N     # Character to use in consensus for uncovered positions

This information has been added in lines 137-138 of the manuscript.

6. Figure 1: the "Positive sgRNA" and "Negative sgRNA" labels should indicate that they referred to samples collected at the last day of remdesivir dosing.

Corrected.

7. Lines 157-160: What do the numbers in parenthesis refer to, IQR or range or something else? Please clarify.

It refers to the interquartile range (quartile 1; quartile 3). We corrected in the manuscript.

8. The work would benefit from English editing.

Thank you for your suggestion, this article was corrected by a native English editor called Donna Pringle. We actually added it in the acknowledgment section (lines 279-280) but we will check again and correct English grammar.

**Reviewer #3 (Comments for the Author):**

This is a very interesting paper and I feel that it adds to the body of work surrounding COVID-19. Here are some suggestions:

Line 27: Delete 'of', it should read "No evidence of global widespread 28 RDV-resistance mutations has been reported"

Corrected.

Line 28 - 30: Change wording from 'or' into 'to'. "Determining emergent mutations prior to..."

The actual correction should be prior and subsequent antiviral therapy, as we sequence both clinical samples before and after remdesivir therapy. We corrected in the manuscript.
Line 32: Why is there a (63.2%) in the sentence. Needs clarification.

Thank you for your correction. The percentages indicate non-responder patients among the total patients of each subgroup: 36 of 111 patients receiving 5 days of RDV, and 21 of 38 patients receiving between 6-20 days of RDV. We corrected it in the line 33.

Line 33 -34: Clarify Next Generation Sequencing. 454 Pyrosequencing or Illumina, or some other type?

We performed next generation sequencing using the Illumina platform as indicated in the method section, but we now also corrected it in the abstract.

Line 40 - 42: Try not to use 'and' two times in one sentence. Substitute one of the 'and' for 'as well as'

Corrected.

Line 47: Capitalize Remdesivir or use RDV since it was defined earlier.

Thank you for your correction. The name of drugs must be written in lower case letters, however, we substituted it for RDV in this case. Thank you.

Line 49: Capitalize Remdesivir or use RDV since it was defined earlier.

Corrected.

Line 79: Capitalize the drug names. They are proper nouns.

As we mention the name of drugs must be written in lower case letters, they are not proper names and are written in such a way by the FDA.

U.S. Food and Drug Administration. Coronavirus (COVID-19) update: FDA Approves First Treatment for COVID-19. (2020 October 22) Available from: https://www.fda.gov/news-events/press-announcements/fda-approves-first-treatment-covid-19.

Line 89: Add a comma after in vitro

Corrected.

Line 90: Add a comma after V557L

Corrected.

Line 116: Clarify Next Generation Sequencing. 454 Pyrosequencing, Illumina, or some other type?

We added in that section that the platform used was Illumina.

Line 157: Clarify what (54;73) means. Is this 54 - 73 years old?

It refers to the interquartile range (quartile 1; quartile 3). We corrected in the manuscript.

Line 178: Remove the second 'only' in the sentence. It should read, "The only non-synonym mutation detected..."

Corrected.
Line 181: Try to avoid using 'and' two times in one sentence. Change the 'and' to 'as well as'
Corrected.

Line 194: Be constant with your italics of nsp12.
Thanks for the correction, we used italics when it refers to the gene sequence, but not when
refers to the protein.

Line 196: Be constant with your italics of nsp12.
In this case, it should not be in italics as it refers to the protein and the different residues
implicated in the interaction between the nsp12 protein and the drug.

Line 195-198: Run-on sentence. Break into two sentences if possible.
It is a long sentence because we name all residues implicated in the interaction, however, we
cannot break it into two sentences as it will lose the sense of the sentence.

Line 207: Be constant with your italics of nsp12.
In this case, it should not be in italics as it refers to the protein.

Line 209: Be constant with your italics of nsp12.
In this case, it should not be in italics as it refers to the protein.

Line 213 - 217: Avoid using 'and' multiple times in a sentence. Break this sentence into 1-3
sentences because it is too long.
Thanks for your comment. I broke it into 3 different sentences.

Line 410: Make sure that in Table 1, all of your Clinical Characters are Capitalized. Be consistent.
Corrected.
September 2, 2022

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Re: Spectrum02448-22R1 (Genetic study of SARS-CoV-2 nsp12 in non-responder COVID-19 patients to remdesivir)

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Supplemental Material: Accept