Antiviral treatments lead to the rapid accrual of hundreds of SARS-CoV-2 mutations in immunocompromised patients

Authors: Nicholas M. Fountain-Jones[1,2*], Robert Vanhaeften[1], Jan Williamson[1], Janelle Maskell[1], I-Ly J Chua[1], Michael Charleston[2] & Louise Cooley[1,3]

Affiliations:

[1] Royal Hobart Hospital, Pathology Department, Hobart Australia 7001.

[2] School of Natural Sciences, University of Tasmania, Hobart Australia 7001.

[3] School of Medicine, University of Tasmania, Hobart Australia 700.1

Keywords: Paxlovid, Molnupiravir, Lageviro, virus evolution, resistance

Abstract

The antiviral Molnupiravir (Lageviro) is widely used across the world to treat SARS-CoV-2 infection. Molnupiravir reduces viral replication by inducing mutations throughout the genome, yet in patients that do not clear the infection, the longer-term impact of the drug on virus evolution is unclear. Here, we used a case-control approach to monitor SARS-CoV-2 genomes through time in nine immunocompromised -patients with five treated with Molnupiravir. Within days of treatment, we detected a large number of low-frequency mutations in patients and that these new mutations could persist and, in some cases, were fixed in the virus population. All patients treated with the drug accrued new mutations in the spike protein of the virus, including non-synonymous mutations that altered the amino acid sequence. Our study demonstrates that this commonly used antiviral can 'supercharge' viral evolution in immunocompromised patients, potentially generating new variants and prolonging the pandemic.

Main text:

Persistent SARS-CoV-2 infection in immunocompromised patients is recognized as an important source of genomic variation and is linked to the evolution of novel variants [1–3]. How commonly used antivirals such as Molnupiravir shape viral evolution is an important knowledge gap. As of December 2022, Molnupiravir is routinely used globally to treat COVID patients in and outside of hospital settings, including treatment of immunocompromised patients [4]. Molnupiravir and other similar direct-acting SARS-CoV-2 antivirals promote mutagenesis by incorporating the prodrug NHC (β-D-N4-hydroxycytidine) into the virus-dependent RNA polymerase (RdRp)[5]. When the RdRp uses the NHC modified RNA as a template it promotes an 'error catastrophe' [5,6] that inhibits the replication of SARS-CoV-2. For immunocompromised patients that do not clear infection after antiviral treatment, the impact that these drugs have on patterns of virus evolution is unclear.

We intensively monitored nine immunocompromised patients; five patients were swabbed pre and post Molnupiravir treatment and four patients did not receive the drug. We found that as little as 10 days after Molnupiravir treatment, patient SARS-CoV-2 genomes had accrued on average 30 new low-mid frequency variants (between 10-90% of reads, Fig 1). We did not find similar changes to viral diversity in the patients not treated with Molnupiravir (Fig. 1). We commonly detected non-synonymous mutations in the spike protein that is the current vaccine target (Fig. 1, Appendix 1) and subsequent samples from these patients demonstrated that some of these spike mutations were fixed (frequency > 90%, e.g., Patient E Thr547Lys). In Patient B, 36 days after treatment 10 novel non-synonymous mutations were fixed, including mutations in the spike protein (Fig. 1/S1). This represents approximately 33% of the mutations that define the Omicron variant [7]. However, we found some mutations were

transient, as many were not detected in the subsequent samples (Fig. 1, multiple infections were ruled out).

These data highlight the risk of treating immunocompromised patients with error generating antivirals such as Molnupiravir. All of the individuals in our study remained persistently PCR positive post-treatment, although active monitoring for clearance was not undertaken by the institution. It is possible they were infectious in the hospital and in their communities, and onward transmission of these highly divergent viruses is likely. This commonly used class of antivirals has the capability to supercharge SARS-CoV- 2 evolution, and uncontrolled use may generate new variants with a transmission advantage that prolongs the pandemic and makes other therapeutics less effective.

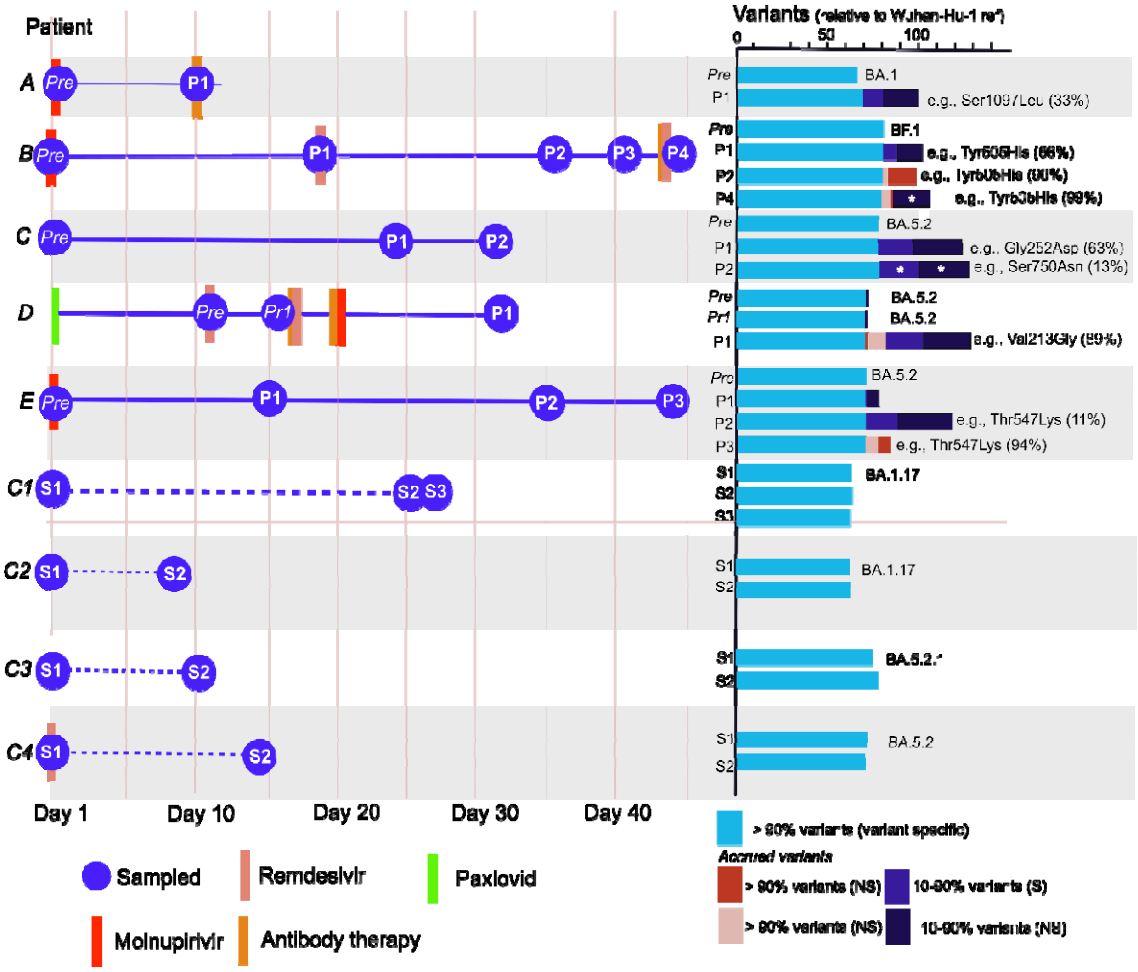


Fig. 1: Patient histories, sampling regimen and variant count in each sample (Prep: pre-treatment sample, pr1: pre Molnupirivir treatment but post Remdesivir treatment, P1-4: subsequent samples post-treatment, S1-3: samples from untreated patients). The Omicron subvariant lineage of each patient is reported next to the variant count from the pre-sample. Patients with dotted lines (C1-4) were not treated with Molnupirivir. The text next to the variant counts highlights a non-synonymous mutation to the spike protein that had either the highest frequency or increased in frequency in subsequent samples. White star: variants found were completely distinct from the previous sample.

Data availability statement

All data will be available online at GISAID. The sequence alignment is available upon request.

Funding

This project was supported by an Australian Research Council Discovery Project Grant (DP190102020).

References

1. Martin DP, Lytras S, Lucaci AG, et al. Selection analysis identifies unusual clustered mutational changes in Omicron lineage BA.1 that likely impact Spike function [Internet]. 2022 [cited 2022 Aug 30];2022.01.14.476382. Available from: https://www.biorxiv.org/content/10.1101/2022.01.14.476382v1

2. Karim F, Moosa MYS, Gosnell BI, et al. Persistent SARS-CoV-2 infection and intra-host evolution in association with advanced HIV infection [Internet]. 2021 [cited 2022 Aug 29];2021.06.03.21258228. Available from: https://www.medrxiv.org/content/10.1101/2021.06.03.21258228v1

3. Tegally H, Wilkinson E, Giovanetti M, et al. Detection of a SARS-CoV-2 variant of concern in South Africa. Nature 2021;592(7854):438–43.

4. Singh AK, Singh A, Singh R, Misra A. An updated practical guideline on use of molnupiravir and comparison with agents having emergency use authorization for treatment of COVID-19. Diabetes Metab Syndr 2022;16(2):102396.

5. Kabinger F, Stiller C, Schmitzová J, et al. Mechanism of molnupiravir-induced SARS-CoV-2 mutagenesis. Nat Struct Mol Biol 2021;28(9):740–6.

6. Sheahan TP, Sims AC, Zhou S, et al. An orally bioavailable broad-spectrum antiviral inhibits SARS-CoV-2 in human airway epithelial cell cultures and multiple coronaviruses in mice. Sci Transl Med 2020;12(541):eabb5883.

7. Fantini J, Yahi N, Colson P, Chahinian H, La Scola B, Raoult D. The puzzling mutational landscape of the SARS-2-variant Omicron. Journal of Medical Virology 2022;94(5):2019–25.

8. Turakhia Y, Thornlow B, Hinrichs AS, et al. Ultrafast Sample placement on Existing tRees (UShER) enables real-time phylogenetics for the SARS-CoV-2 pandemic. Nat Genet 2021;53(6):809–16.

Supplementary text:

Methods

Study population

The 9 patients included in this study were immunocompromised due to a number of aetiologies, including haematological malignancy, solid organ and allogenic stem cell transplantation, and treatment of vasculitis that had tested qPCR positive to SARS CoV-2 infection. Six patients were treated with antivirals (one just with Remdesivir) and five were treated with Molnupirivir. The patients not treated with Molnupirivir were diagnosed with the virus before approval of the drug in Australia and acted as a quasi-control.

Real-Time PCR assay

Flocked swabs were collected from the throat and nasopharynx and immediately placed in 2.5mL viral transport Fluid (VTF).

Samples were vortexed for 1 minute and 200µL VTF was extracted on the Magnapure96 instrument (Roche) using the DNA/RNA SV kit and Pathogen Universal 200 protocol.

Ten microlitres of eluate was used in a 20µL multiplex real time PCR containing 1x MDX-016 mastermix (Meridian Bioscience), with primers and probes (Bioneer) targeting the SARS-CoV-2 matrix and RdRP genes, and human RNaseP gene as a control. Oligo sequences are shown below.

Oligo Name	Sequence (5'-3')	Final concentration (µM)
RHH-M-F	TGTTGCTACATCACGAACGC	0.25µM
RHH-M-R	GCCAATCCTGTAGCGACTGT	0.25µM
RHH-M-Pr	FAM-AACCTGAGTC[EBQ]ACCTGCTACACG-EBQ	0.1µM
RHH-RdRP-F	CGTGTTGTAGCTTGTCACACC	0.25µM

RHH-RdRP-R	ATAGTGAACCGCCACACATGA	0.25μM
RHH-RdRP-P	HEX-TCTATAGATT[EBQ]AGCTAATGAGTGTGCTCA-EBQ	0.1μM
RNaseP-F	AGATTTGGACCTGCGAGCG	0.1μM
RNaseP-R	GAGCGGCTGTCTCCACAAGT	0.1μM
RNaseP-Pr	CY5-TTCTGACCTG[EBQ]AAGGCTC-EBQ	0.1μM

Table S1. RT-PCR primer and probe sequences.

PCR was performed on an LC480 instrument (Roche) with thermal cycling parameters as follows: 50°C for 15 minutes, 95°C for 2 minutes, then 40 cycles of 95°C for 10 seconds and 60°C for 30 seconds with fluorescence collected at the 60°C step.

Tiled amplicon sequencing

Overlapping amplicons were generated using the QIAseq SARS-CoV-2 Primer Panel kit (Qiagen) as per the manufacturer's instructions except substituting a custom primer set and using a 60°C annealing/extension temperature.

Amplicon	Pool	Oligo Name	Sequence	Length	RefSeq 5'	RefSeq 3'	Amplicon size	Final Concentration in PCR
1	1	RHH_SARSCOV2_01F	TCCCAGGTAACAAACCAACCAA	22	18	39	1102	0.04uM
	1	RHH_SARSCOV2_01R	TGAATAGTCTTGATTATGGAATTTAAG	30	1090	1119		0.04uM
2	2	RHH_SARSCOV2_02F	ACCTTCAATGGGGAATGTCCA	21	1058	1078	1112	0.04uM
	2	RHH_SARSCOV2_02R	AACTTCTCTTCAAGCCAATCAAGG	24	2146	2169		0.04uM
3	1	RHH_SARSCOV2_03F	TGGCTAACTAACATCTTTGGCAC	23	2099	2121	1145	0.08uM
	1	RHH_SARSCOV2_03R	CCGTCTTGTTGACCAACAGTTT	22	3222	3243		0.08uM
4	2	RHH_SARSCOV2_04F	ACCTGAAGAAGAGCAAGAAGAAGA	24	3175	3198	1127	0.04uM
	2	RHH_SARSCOV2_04R	GCACTGTCTTTGCCTCCTCT	20	4282	4301		0.04uM
5	1	RHH_SARSCOV2_05F	GGGTGTTTTAACTGCTGTGGT	21	4144	4164	1134	0.04uM
	1	RHH_SARSCOV2_05R	TCTGCCCATTTAATAGAAGTTAAACCA	28	5250	5277		0.04uM
6	2	RHH_SARSCOV2_06F	ACACTAAAAAGTGGAAATACCCACAA	26	5220	5245	1160	0.04uM
	2	RHH_SARSCOV2_06R	ATTATCCATTCCCTGCGCGT	20	6360	6379		0.04uM
7	1	RHH_SARSCOV2_07F	CGTTTGATGTACTGAAGTCAGAGG	24	6336	6359	1021	0.04uM
	1	RHH_SARSCOV2_07R	AGCCAAGAATTACTAATAAAATGTACT	29	7328	7356		0.04uM
8	2	RHH_SARSCOV2_08F	TGGATTGGCTGCAATCATGC	20	7288	7307	1111	0.04uM
	2	RHH_SARSCOV2_08R	CAAAGCAATGTTGTGACTTTTTGCT	25	8374	8398		0.04uM
9	1	RHH_SARSCOV2_09F	GCGTCATATTAATGCGCAGGT	21	8353	8373	1097	0.04uM
	1	RHH_SARSCOV2_09R	AGGCAAGGCATGTTACTACGA	21	9429	9449		0.04uM
10	2	RHH_SARSCOV2_10F	AGCATCTATAGTAGCTGGTGGTA	23	9397	9419	1141	0.04uM
	2	RHH_SARSCOV2_10R	GTAACAAAAAGAGACACAGTCATAAT	28	10510	10537		0.04uM
11	1	RHH_SARSCOV2_11F	TGGTTCATGTGGTAGTGTTGGT	22	10480	10501	1153	0.08uM
	1	RHH_SARSCOV2_11R	AAAATAGCCTAAGAAACAATAAACTA	29	11604	11632		0.08uM
12	2	RHH_SARSCOV2_12F	TGCCCTATTTTCTTCATAACTGGT	24	11561	11584	1117	0.04uM
	2	RHH_SARSCOV2_12R	TGACAGCAGAATTGGCCCTT	20	12658	12677		0.04uM
13	1	RHH_SARSCOV2_13F	GCATGGCCTCTTATTGTAACAGC	23	12632	12654	1149	0.04uM
	1	RHH_SARSCOV2_13R	TATGTGGTACCATGTCACCGTC	22	13759	13780		0.04uM

14	2	RHH_SARSCOV2_14F	GTCCAGCTGTTGCTAAACATGA	22	13718	13739	1124	0.04uM
	2	RHH_SARSCOV2_14R	TCTGATATCACACATTGTTGGTAGAT	26	14816	14841		0.04uM
15	1	RHH_SARSCOV2_15F	GCTCAGGATGGTAATGCTGC	20	14767	14786	1118	0.04uM
	1	RHH_SARSCOV2_15R	TGAGAGCAAAATTCATGAGGTCC	23	15862	15884		0.04uM
16	2	RHH_SARSCOV2_16F	ATGTTGGACTGAGACTGACCTT	22	15834	15855	1104	0.04uM
	2	RHH_SARSCOV2_16R	GGCATTACTGTATGTGATGTCAGC	24	16914	16937		0.04uM
17	1	RHH_SARSCOV2_17F	ACCGAGGTACAACAACTTACAAA	23	16868	16890	1112	0.04uM
	1	RHH_SARSCOV2_17R	AAGGTCTCTATCAGACATTATGCAAA	27	17953	17979		0.04uM
18	2	RHH_SARSCOV2_18F	GCTATTACCAGAGCAAAAGTAGGC	24	17926	17949	1125	0.04uM
	2	RHH_SARSCOV2_18R	AGCTTTAGGGTTACCAATGTCGT	23	19028	19050		0.04uM
19	1	RHH_SARSCOV2_19F	AGCAGACAAATTCCCAGTTCTTC	23	19005	19027	1130	0.04uM
	1	RHH_SARSCOV2_19R	CGGCTTCTCCAATTAATGTGACT	23	20112	20134		0.04uM
20	2	RHH_SARSCOV2_20F	AACCATCTGTAGGTCCCAAACA	22	20075	20096	1120	0.04uM
	2	RHH_SARSCOV2_20R	TCAGCATTCCAAGAATGTTCTGTT	24	21171	21194		0.04uM
21	1	RHH_SARSCOV2_21F	GCTAGCTCTTGGAGGTTCCG	20	21138	21157	1132	0.04uM
	1	RHH_SARSCOV2_21R	GTGATGTTAATACCTATTGGCAAATCT	27	22243	22269		0.04uM
22	2	RHH_SARSCOV2_22F	CCCTCAGGGTTTTTCGGCTT	20	22210	22229	1089	0.04uM
	2	RHH_SARSCOV2_22R	GGATCACGGACAGCATCAGT	20	23279	23298		0.04uM
23	1	RHH_SARSCOV2_23F	GGCAGAGACATTGCTGACACT	21	23258	23278	1131	0.04uM
	1	RHH_SARSCOV2_23R	TGCTGTGGAAGAAAGTGAGTCT	22	24367	24388		0.04uM
24	2	RHH_SARSCOV2_24F	TGAGAACCAAAAATTGATTGCCAAC	25	24313	24337	1115	0.04uM
	2	RHH_SARSCOV2_24R	GTTCCAATTGTGAAGATTCTCATAAAC	28	25400	25427		0.04uM
25	1	RHH_SARSCOV2_25F	CAGTGCTCAAAGGAGTCAAATTACA	25	25350	25374	1130	0.04uM
	1	RHH_SARSCOV2_25R	AGTTCGTTTAGACCAGAAGATCAG	24	26456	26479		0.04uM
26	2	RHH_SARSCOV2_26F	ACGTTTACTCTCGTGTTAAAAATCTGA	27	26414	26440	1030	0.04uM
	2	RHH_SARSCOV2_26R	AGCTCACAAGTAGCGAGTGT	20	27424	27443		0.04uM
27	1	RHH_SARSCOV2_27F	TGAAAATTATTCTTTTCTTGGCACTGA	27	27395	27421	1135	0.04uM
	1	RHH_SARSCOV2_27R	AGCCAATTTGGTCATCTGGACT	22	28508	28529		0.04uM
28	2	RHH_SARSCOV2_28F	AATTCCCTCGAGGACAAGGC	20	28467	28486	1127	0.08uM
	2	RHH_SARSCOV2_28R	CGTAAACGGAAAAGCGAAAACG	22	29572	29593		0.08uM
29	1	RHH_SARSCOV2_29F	CCTGCTAGAATGGCTGGCAA	20	28892	28911	979	0.04uM
	1	RHH_SARSCOV2_29R	GTCATTCTCCTAAGAAGCTATTAAAAT	29	29842	29870		0.04uM

Table S2. Tiled amplicon primer sequences.

Amplicon concentration was measured using the Qubit 1X BR kit on a Qubit flex instrument (Thermo). Approximately 200ng of amplicon was fragmented and barcoded using the Illumina DNA Prep kit as per the manufacturer's instructions before 2x151 sequencing on an Illumina Miniseq. Basecalling, demultiplexing and adapter trimming were performed on-instrument.

Sequence analysis and variant calling

Raw reads were imported in CLC Genomics Workbench 21.0.5 and analyzed using a custom workflow. Reads were quality trimmed (<20) before being aligned to the SAR CoV-2 Wuhan-Hu-1 reference sequence. Primer sequences were trimmed from read mappings and variants called using the low frequency variant caller with a threshold of 10% VAF, a minimum coverage of 10-fold and a minimum of 5 variant read support. A consensus sequence was derived from mapped reads with bases below 10-fold coverage replaced with N

and mixed base sites between 10% and 90% given IUB mixed base codes. Lineage and sub-lineages were identified through the web base Pangolin version 3.1.20 version date 28/02/2022.

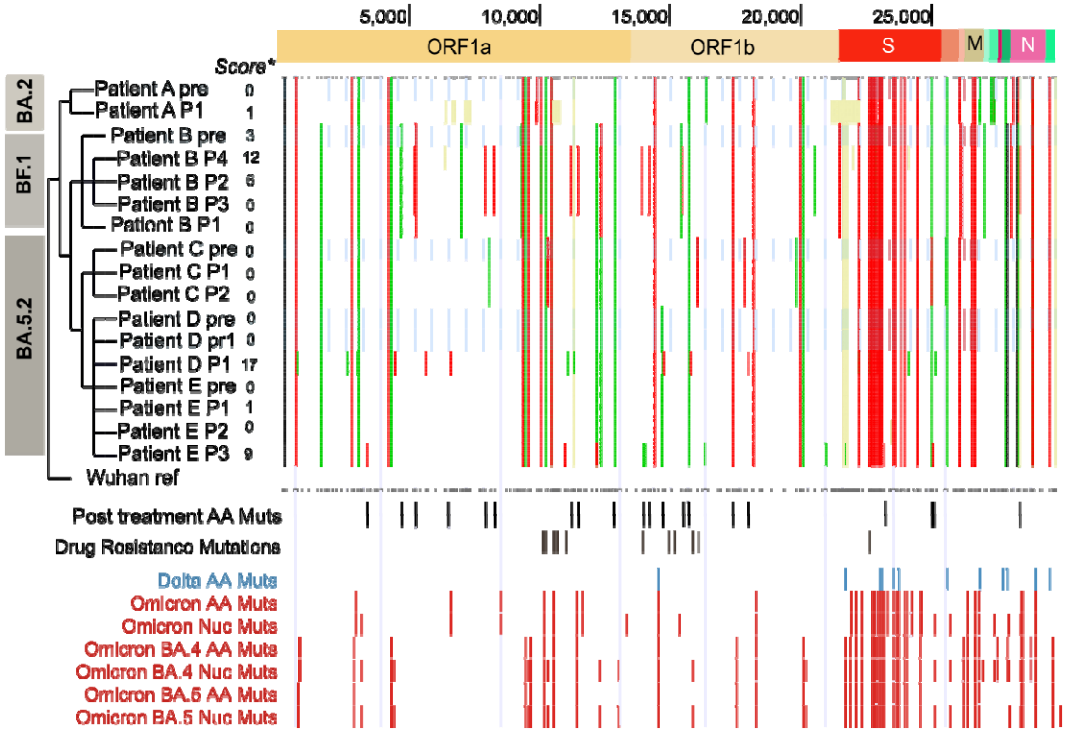
To compare variants from our patient samples with global reference sequences and to visualize the locations of the mutations across the genome we used the Ultrafast Sample placement on Existing tRee (UShER) pipeline [8] and the University of California Santa Cruz (UCSC) genome viewer (https://genome.ucsc.edu/index.html).

Supplementary results

The variants that accrued in patients after treatment with Lageviro tended to be scattered across the genome and included mutations not commonly sampled in global Omicron genomes (Fig. S1). The accrued mutations we detected in the spike (s) gene tended to be clustered in two locations (Fig. S1), but the functional relevance of the mutations was unclear. While we did not detect any known drug resistance mutations, we did detect non-synonymous mutations in ORF 1b in the neighbouring amino acids (Fig. S1).

The UShER analysis showed that while most samples from individual patients clustered together there were potentially novel or rare mutations in the sequences post-treatment (Fig. 1). There were samples with so many mutations that they were phylogenetically distinct and difficult to place on the global SARS CoV-2 phylogeny. For example, the genomes from the 4th sample from Patient B (P4) and the first sample from patient D (P1) were so distinct that the minimum number of mutations needed to add the sample to the global tree was 14 and 17

177 respectively (Fig. 1). While the first sample taken from Patient A (P1) could be added to the
178 global reference tree without requiring any new mutations, these mutations accrued were
179 phylogenetically distinct indicating that the sample was phylogenetically distinct from the
180 pre-treatment sample prior and to the other samples taken post treatment (Fig. 1).



181

182 **Fig. S1:** Waterfall plot showing the SARS CoV-2 genome coordinates of the nonsynonymous
183 (amino acid (AA), red) and synonymous mutations (nucleotide (Nuc), green) across the
184 treated patients in this study (top panel). Mid panels = Location of non-synonymous
185 mutations just detected in treated patients with the location of known resistance mutations
186 below. Bottom panels: Mutations defining omicron and BA.4/5. Light blue bars: Samples
187 taken prior to Molnupiravir treatment. We used the Ultrafast Sample placement on Existing
188 tRee (UShER) pipeline [8] to generate lineage assignments, phylogenetic placement and
189 parsimony scores (left panel). The parsimony score is based on the minimum number of
190 additional mutations needed to place the sequence in the reference tree. Wuhan ref = Wuhan
191 reference sequence (MN908947.3).

192

193