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RESEARCH ARTICLE

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Molnupiravir increases SARS-CoV-2 genome diversity and complexity: A case-control cohort study

Cesare Ernesto Maria Gruber[1] | Fabio Giovanni Tucci[2] | Emanuela Giombini[1] | Valentina Mazzotta[3] | Pietro Giorgio Spezia[1] | Martina Rueca[1] | Ilaria Mastrorosa[3] | Lavinia Fabeni[1] | Giulia Berno[1] | Ornella Butera[4] | Silvia Rosati[3] | Eliana Specchiarello[1] | Fabrizio Carletti[1] | Daniele Focosi[5] | Emanuele Nicastri[3] | Enrico Girardi[6] | Andrea Antinori[3] | Fabrizio Maggi[1]

[1]Laboratory of Virology, National Institute for Infectious Diseases Lazzaro Spallanzani IRCCS, Rome, Italy

[2]Department of Biology, University of Rome Tor Vergata, Rome, Italy

[3]Clinical and Research Infectious Diseases Department, National Institute for Infectious Diseases Lazzaro Spallanzani IRCCS, Rome, Italy

[4]Laboratory of Microbiology, National Institute for Infectious Diseases Lazzaro Spallanzani IRCCS, Rome, Italy

[5]North-Western Tuscany Blood Bank, Pisa University Hospital, Pisa, Italy

[6]Scientific Direction, National Institute for Infectious Diseases "L. Spallanzani"—IRCCS, Rome, Italy

Correspondence

Martina Rueca, Laboratory of Virology, National Institute for Infectious Diseases Lazzaro Spallanzani IRCCS, Rome, Italy.
Email: martina.rueca@inmi.it

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Abstract

Molnupiravir, an oral direct-acting antiviral effective in vitro against SARS-CoV-2, has been largely employed during the COVID-19 pandemic, since December 2021. After marketing and widespread usage, a progressive increase in SARS-CoV-2 lineages characterized by a higher transition/transversion ratio, a characteristic signature of molnupiravir action, appeared in the Global Initiative on Sharing All Influenza Data (GISAID) and International Nucleotide Sequence Database Collaboration (INSDC) databases. Here, we assessed the drug effects by SARS-CoV-2 whole-genome sequencing on 38 molnupiravir-treated persistently positive COVID-19 outpatients tested before and after treatment. Seventeen tixagevimab/cilgavimab-treated outpatients served as controls. Mutational analyses confirmed that SARS-CoV-2 exhibits an increased transition/transversion ratio seven days after initiation of molnupiravir. Moreover we observed an increased G->A ratio compared to controls, which was not related to apolipoprotein B mRNAediting enzyme, catalytic polypeptide-like (APOBEC) activity. In addition, we demonstrated for the first time an increased diversity and complexity of the viral quasispecies.

KEYWORDS

complexity, diversity, molnupiravir, quasispecies, SARS-CoV-2, tixagevimab/cilgavimab, transition, transversion

Cesare Ernesto Maria Gruber and Fabio Giovanni Tucci contributed equally to this study.

1 | INTRODUCTION

Molnupiravir (MK-4482/EIDD-2801) is an oral antiviral prodrug with broad activity against RNA viruses that was authorized at the end of 2021 for the treatment of SARS-COV-2 outpatients at high risk of progression to severe COVID-19 disease.[1–3] Molnupiravir targets the RNA-dependent RNA polymerase (RdRp) enzyme, which is responsible for replicating the SARS-CoV-2 genome.[4] Molnupiravir acts as a ribonucleoside analog (β-D-N4-hydroxycytidine (NHC)−5′-isopropyl ester) that, in its active form (MTP) is used as an alternative substrate by the RdRp, interfering with replication and introducing mutations into viral RNA. When viral RNA nucleotides G or A are present, MTP is frequently incorporated instead of C or U. Therefore, in later replication, MTP present in the template strand is replaced with A or U in the copy RNA, introducing transition errors (C to U; U to C; G to A; A to G).[5] Despite company-sponsored trials showed safety and efficacy,[6–8] a subsequent larger randomized controlled trial in mostly vaccinated people found no reduction in hospitalizations, leading the European Medicines Agency to deauthorize it.[9] At the same time, an analysis of the Global Initiative on Sharing All Influenza Data (GISAID) and the International Nucleotide Sequence Database Collaboration (INSDC) databases suggested that molnupiravir-generated variants could be transmissible and fit.[10] Although such mutagenesis is recognized as an antiviral strategy,[11] these findings suggest further investigations.

Normally, the transitions-to-transversions ratio is about 2:1 for SARS-CoV-2,[12,13] while molnupiravir typically induces a 14:1 ratio.[4,14] To discriminate whether the increased number of transitions is due to molnupiravir activity or rather to apolipoprotein B mRNAediting enzyme, catalytic polypeptide-like (APOBEC) antiviral activity[15] an analysis of specific surrounding motifs is required.[16]

The detection of viral quasispecies has been widely used to assess pathogen evolution during antiviral treatment,[17] and investigations on intrahost variability and its effects on clinical manifestations have been extensively carried out in immunocompromised patients treated with drugs other than molnupiravir.[18,19] The dynamics of short-term intrahost mutations of SARS-CoV-2 during molnupiravir treatment have been previously described in small cohorts of high-risk but generally immunocompetent outpatients,[20,21] but it remains unknown whether molnupiravir-generated SARS-CoV-2 variants can persist in immunocompromised patients.[22] In particular, viral quasispecies diversity (defined as the genetic distance among the strains present at a given time in the quasispecies) and viral quasispecies complexity (defined as the number of strains present at a given time in the quasispecies)[23] have not been reported yet in molnupiravir-treated patients.

Here, we analyzed a cohort of 38 molnupiravir-treated but persistently positive COVID-19 immunocompetent and immunocompromised outpatients infected with BA.5* VOC and at high risk of COVID-19 progression that, after 7 days of therapy, had not cleared the virus. The number and location (positioning and surrounding motif) of single-nucleotide polymorphisms (SNP) that occurred on Day 7 were assessed, as well as the prevalence of G->A transitions, and the transition/transversion ratio. Finally, we analyzed the intrahost diversity and complexity of the spike gene in the viral quasispecies to reveal how the drug impacted the intrahost genetic variability of the virus, compared to a control group treated with a different drug (monoclonal antibody) targeting the spike gene only.

2 | MATERIALS AND METHODS

2.1 | Patients and specimens

A total of 631 SARS-CoV-2 positive patients with a high risk of COVID-19 progression attending the outpatients service for early treatment of COVID-19 of the National Institute for Infectious Diseases (INMI) "Lazzaro Spallanzani" in Rome between July 2022 and September 2022 were treated with molnupiravir monotherapy (800 mg orally twice a day for 5 days). All patients, before initiation of the study, did not receive any previous antiviral treatment. Moreover, 198 of these outpatients resulted positive for SARS-CoV-2 at Day 7 after treatment initiation; 38 of them (15 females and 23 males; median age of 76, 95% confidence interval [CI]: 62–83, and range of 35–92 years) were retrospectively recruited on the basis of viral load at Days 0 and 7 after treatment initiation and the availability of residual samples, once all the required diagnostic tests had been carried out.

In more detail, enrolled patients were treated on average 2.2 (standard deviation: 0.92) days after symptoms onset, and 97% of them had no history of prior SARS-CoV-2 infection. Immunocompetent individuals represented 58% of patients, and 42% of them were immunocompromised due to primary or secondary immunodeficiency.

Almost all patients recovered within 30 days from symptom onset, except for one patient who required hospitalization after treatment.

As a control group, a historical cohort of 17 patients (13 females and four males; median age of 72, 95% CI: 61–79, and range of 54–85 years) treated with 300/300 mg of tixagevimab/cilgavimab (Evulsheld®) monotherapy was randomly selected among the 22 subjects previously included in another study.[18] This cocktail of two antispike (S) monoclonal antibodies are known not to be mutagenic but able to select S-mutants only.[24] The baseline characteristics of the two cohorts of patients are summarized in Table 1.

Nasopharyngeal swabs (NPS) were collected from all patients before treatment (T0) and 7 days after treatment initiation (T7). SARS-CoV-2 RNA detection was performed by a commercial real-time polymerase chain reaction (PCR) assay (Alinity m® SARS-CoV-2 Assay; Abbott). SARS-CoV-2 loads were estimated using the cycle threshold (Ct) value obtained by amplification.

2.2 | Sequencing and genetic analysis of SARS-CoV-2 genome

Whole genome sequencing (WGS) was performed on available residual NPS samples. After nucleic acid extraction by the QiaSymphony®

TABLE 1 Demographic and virologic characteristics of the study populations, grouped by type of treatment.

Parameter	Molnupiravir-treated patients ($n = 38$)	Tixagevimab/cilgavimab - treated patients ($n = 17$)	p Value
Age, median (IQR)	76 (62–83)	72 (61–79)	0.40
Gender (%)			**0.02**
Male	60.5	23.5	
Female	39.5	76.5	
SARS CoV-2 Ct values, median (IQR)			
At Day 0	12.9 (12.1–14.8)	16.2 (12.8–17.6)	0.20
At Day 7	21.4 (18.3–24)	22 (19.0–25.5)	0.98
Anti-S IgG levels at Day 0 (BAU/mL), median (IQR)	307.9 (45.7–1084.4)	477 (151–885.1)	0.23
Immunocompromised patients (%)	42.1	35.3	0.77
Patients with oncohematological disorders (%)	10.4	29.4	0.18
Vaccinated patients (%)	84.6	94.1	0.78
Number of vaccine doses, median (IQR)	3 (3–4)	3 (3–3.3)	0.77

Abbreviations: Ct, cycle threshold; IgG, immunoglobulin G; IQR, interquartile range.

automatic extractor (QIAGEN), libraries for Next Generation Sequencing (NGS) were prepared using the Ion AmpliSeq® SARS-CoV-2 Insight Research Assay, following the manufacturer's instructions (ThermoFisher). Finally, sequencing was carried out on the Ion Torrent Gene Studio S5 Prime sequencer to obtain 5×10^5 reads per sample. Sequenced reads with a mean quality Phred score >20 were selected and trimmed with Trimmomatic v.0.36.[25] Reference-based assembly was performed using the ESCA pipeline[26]; whole genome sequences and high-quality mapping SARS-CoV-2 reads were manually controlled using Geneious 2019.2.3; genome regions with coverage of fewer than five reads were replaced with "N" stretches. The intrasample single-nucleotide variants (iSNVs) were detected using VarScan 2.3.9,[27] and only mutations with a minimum coverage of 20 reads were considered.

After aligning all samples to the Wuhan-Hu-1 sequence (NC_045512.2) as a reference, T0–T7 pairs were compared with each other, analyzing all genome sites with sufficient coverage in both samples (50×). Only variants identified in at least 5% of the reads in the T7 sample, and not present in the T0 sample, were considered.

The nucleotide context of each C->T and G->A transition was also investigated in the molnupiravir-treated samples to evaluate the effect of APOBEC activation in producing transitions. This analysis was performed by using a custom script; the nucleotide context surrounding each C->T and G->A transition in samples treated with molnupiravir and tixagevimab/cilgavimab were systematically examined by evaluating the trinucleotide sequence centered on the mutation position (−1 and +1 bp). Instances of C->T transitions (accompanied by complementary G->A changes) within a [AU]C|G[AU] context were considered potential APOBEC enzyme activity, as reported in previous studies.[15]

Finally, the number of synonymous (silent) nucleotide substitutions per synonymous site and the number of nonsynonymous (amino acid replacement) nucleotide substitutions per nonsynonymous site were calculated using ESCA software[26] and a home-made python script. Mutations were detected by comparing sequence data between T0 and T7 and including all iSNVs with a threshold of 5% minimum frequency.

2.3 | Analysis of SARS-CoV-2 quasispecies diversity and complexity

The genetic diversity of each sample was estimated for the S gene only using the Shannon index (H) based on the allele frequency of each SNV, assuming they are independent of each other:

$$H = -\sum_{i}^{n} P(i)\log_2 P(i),$$

where $P(i)$ is the allele frequency at variable site i. For this analysis, we considered only the SNV with a minimum allele frequency of 5%, supported by at least five reads in position with sufficient coverage (50×). Sites with more than one alternative allele were filtered out.

To assess the number of quasispecies present in molnupiravir- and tixagevimab/cilgavimab-treated patients, the evaluation focused on the amplicons sequenced with the NGS protocol in the S gene only. The quasispecies analysis was conducted individually for each

amplicon's region of the S gene (30 regions in total, with a mean length of 200 bp). For each sample, the highest estimate obtained among all 30 values was considered the minimum number of quasispecies present in the sample. For this purpose, only complete reads spanning the entire amplicon's length were extracted with the Samtools program. CD-HIT software was then used for clustering these reads with an identity threshold of 100% and considering only SNV. Each cluster, supported by a number of reads representing at least 5% of the amplicon's total coverage in both the forward and reverse strands, was finally counted as a quasispecies.

2.4 | Statistical analysis

The results are expressed as median and interquartile range (IQR). The statistical significance of comparisons between molnupiravir and tixagevimab/cilgavimab-treated patients was performed using the Wilcoxon rank-sum test. An independent *t* test was performed to compare the percentage increases between molnupiravir- and tixagevimab/cilgavimab-treated samples. A *p* value less than 0.05 was considered statistically significant in all tests.

3 | RESULTS

3.1 | Temporal kinetics of SARS-CoV-2 NPS loads

SARS-CoV-2 NPS levels were measured in previously untreated COVID-19 outpatients infected with SARS-CoV-2 BA.5* at baseline and Day 7 (T7) since initiation of treatment with either molnupiravir ($n = 38$) or tixagevimab/cilgavimab ($n = 17$). Overall data are shown in Table 1 and Figure 1. Among molnupiravir-treated patients, at T0, SARS-CoV-2 Ct values ranged between 10 and 22 (mean of 13.5), confirming that the levels of the virus extensively differ in individual patients. A substantial decline in mean SARS-CoV-2 loads was evident on Day 7 of treatment when the mean Ct value was 20.9, and the mean Ct drop was 7. Interestingly, although the maximum proportion of molnupiravir-treated patients exhibited a significant decline in SARS-CoV-2 loads (*p* value: 0.0025), few patients had either stable values or slightly decreased Ct values relative to T0, and one patient showed SARS-CoV-2 RNA at higher levels at T7 (Ct 9.0) than at T0 (Ct 17.0).

Among tixagevimab-cilgavimab-treated patients, at T0, SARS-CoV-2 Ct values ranged between 11 and 23 (mean of 15.9); all tixagevimab-cilgavimab-treated patients experienced a significative decline in SARS-CoV-2 loads at Day 7 (mean Ct value: 21.5; range: 16.4–26.5).

3.2 | SNV detection

SARS-CoV-2 detected in NPS samples at T0 and T7 was genetically characterized by whole-genome NGS. All the 55 viral sequences were identified as SARS-CoV-2 BA.5* variant.

When the number of SNV was calculated in samples from molnupiravir-treated patients, a mean of 33.1 (standard deviation: 33.7) was found.

Among these SNV, the median number of transitions per sample was 29.5 (range: 0–160) as shown in Figure 2A. No significant difference between immunocompetent and immunocompromised molnupiravir outpatients was found.

Importantly, the median of SNV transition was significantly lower in tixagevimab/cilgavimab treated patients: two (range: 1–13) (Figure 2B), while median transversion frequencies were similar in molnupiravir and tixagevimab/cilgavimab control patients: 0.5 (range: 0.0–3.0) and 0.5 (range: 0.0–5.0), respectively.

The mean transitions:mutations ratio was higher in molnupiravir-treated patients with respect to the control group: 1.0 (range: 0.0–1.0)

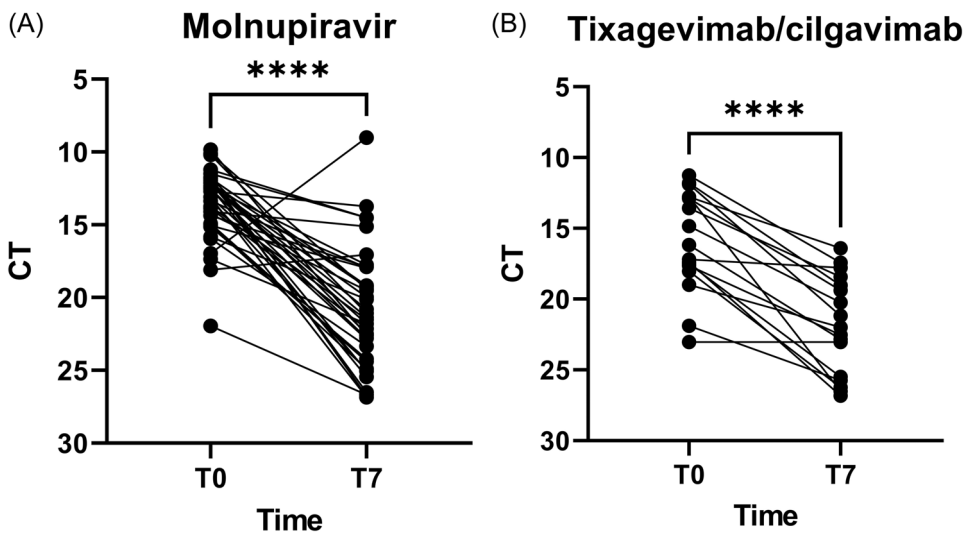

FIGURE 1 SARS-CoV-2 loads at Day 0 (T0) and Day 7 (T7) after molnupiravir treatment initiation (A) and tixagevimab/cilgavimab treatment (B). SARS-COV-2 loads are expressed as Ct values. Each line connects Ct values obtained from a single patient. Ct, cycle threshold.

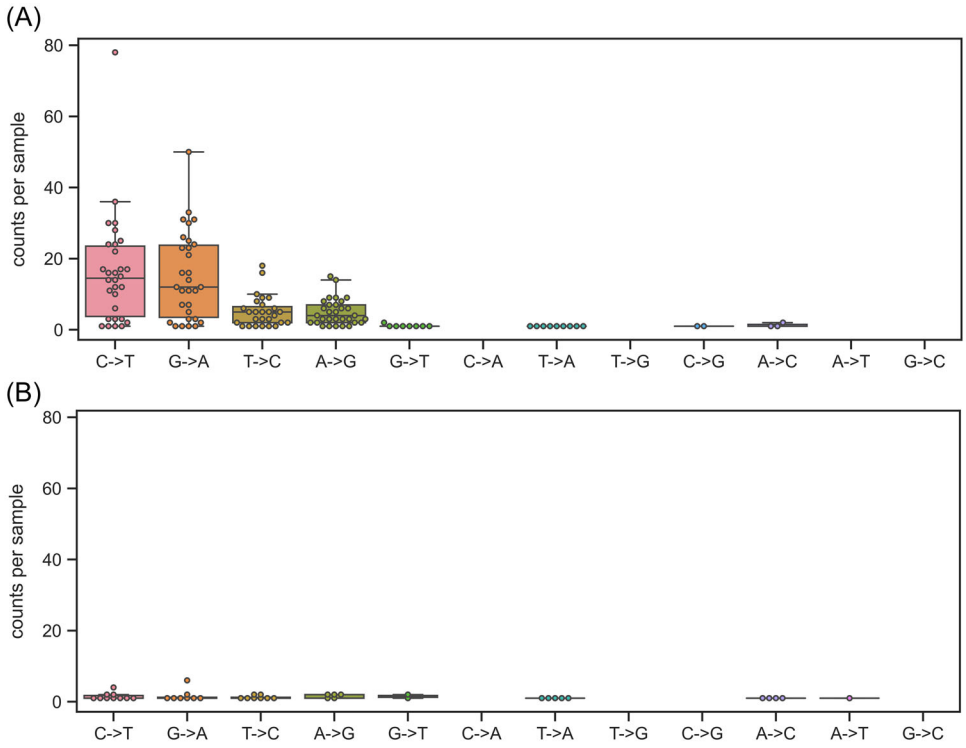

FIGURE 2 Distribution of intrahost single-nucleotide variations (SNV) occurring at minimum frequency of 5% in at least one SARS-CoV-2 sequence at Day 7 compared with sequences at Day 0 after molnupiravir (A) and tixagevimab/cilgavimab (B) treatments.

and 0.9 (range: 0.5–1.0), respectively. In particular, in molnupiravir patients with transitions:mutations ratios near to 1.0, a higher G-to-A mutation ratio was observed (Figure 3).

The contribution of host APOBEC activation in producing the increased number of transitions in the molnupiravir-treated samples was also evaluated, investigating the nucleotide context of each C->T and G->A transition identified. No transitions were found to be associated with APOBEC motifs (data not shown).

Additionally, the SNV distribution was analyzed to assess whether the mutations were restricted to specific regions of the SARS-CoV-2 genome. The analysis revealed that SNV in molnupiravir patients fell uniformly along the viral genome with a median SNVs frequency per gene of 0.002 (range: 0–0.023) (Figure 4A). Similar results were also observed for synonymous and nonsynonymous mutations, having identical medians of 0.001 with ranges respectively of 0–0.008 and 0–0.023 (Figure 5A). Notably, the median normalized frequency of SNV per gene in tixagevimab/cilgavimab-treated patients was 0.001 (range: 0–0.004), as shown in Figure 4B. Finally, medians of synonymous and nonsynonymous mutations in tixagevimab/cilgavimab outpatients was 0.001 with ranges, respectively, of 0–0.004 and 0–0.002 (Figure 5B).

3.3 | Genetic characterization of SARS CoV-2 quasispecies

Firstly, the genetic diversity of SARS-CoV-2 quasispecies was estimated using the Shannon index for the S gene. At T0, a mean Shannon diversity of 3.9 ± 1.7 was found in molnupiravir-treated patients. A significant increase was observed at T7 with a calculated value of 16.7 ± 13.4 ($p < 0.0001$; Wilcoxon rank-sum test). Importantly, the quasispecies diversity remained essentially stable in tixagevimab/cilgavimab-treated patients, ranging from 3.1 ± 1.1 at T0 to 4.1 ± 2.6 at T7 (Figure 6).

Finally, the complexity of SARS-CoV-2 quasispecies was analyzed in each sample. The minimum number of strains was calculated for each of the 30 amplicons of the S gene of the viral genome at T0 and T7. The analysis showed that the number of strains was similar at T0 in patients treated with molnupiravir and tixagevimab/cilgavimab (1.1 ± 0.2 vs. 1.0 ± 0.0, respectively) but differed at T7, increasing in molnupiravir-treated patients but not in tixagevimab/cilgavimab patients (2.2 ± 1.0 vs. 1.3 ± 0.5, respectively) (Figure 7).

4 | DISCUSSION

The finding in the GISAID and ISNDC databases that an increasing number of SARS-CoV-2 sequences characterized by a high transition/transversion ratio[21] was uploaded from many countries after the approval of molnupiravir inspired this study regarding SARS-CoV-2 sequencing from NPS samples collected from 38 patients treated with this prodrug. Since then, web tools have been published to list putative molnupiravir-signed GISAID sequences[28] and to analyze the probability that SARS-CoV-2 mutational spectra stem from molnupiravir action.[29] A recent study on 175 immunocompetent patients

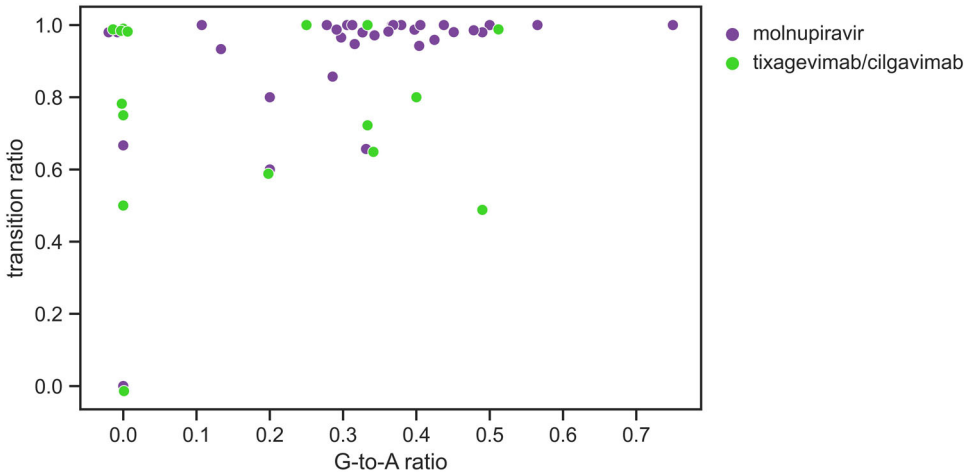

FIGURE 3 Transition ratio versus G->A transition ratio at Day 7 after molnupiravir and tixagevimab/cilgavimab treatments.

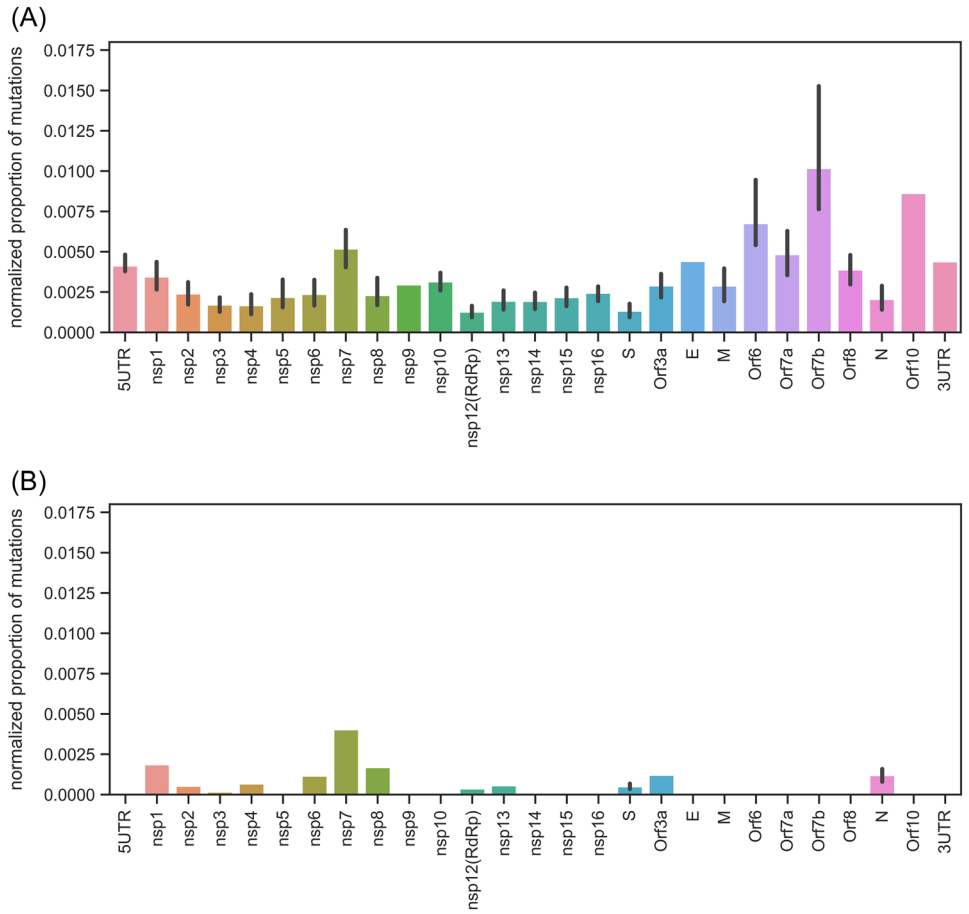

FIGURE 4 Frequency of transitions within each gene of the SARS-CoV-2 genome after molnupiravir (A) and tixagevimab/cilgavimab (B) treatments. The value is normalized according to the base-pair length of each gene 5–95 percentile range is reported with black bars. The envelope (E) and Orf10 genes are represented without bars because, for each of these genes, a single-nucleotide variation was found in a single patient.

followed up for 5 days did not find an infectious virus within 48 h in NPS using culture-PCR,[30] but results might be different in immunocompromised patients, harboring higher basal viral loads. The present study offers important information on the effects of molnupiravir on genetic variability both in immunocompromised and immunocompetent patients. We assess the short-term dynamics of SARS-CoV-2 RNA during the use of molnupiravir and demonstrate that load changes are variable among single individuals. In line with this finding,

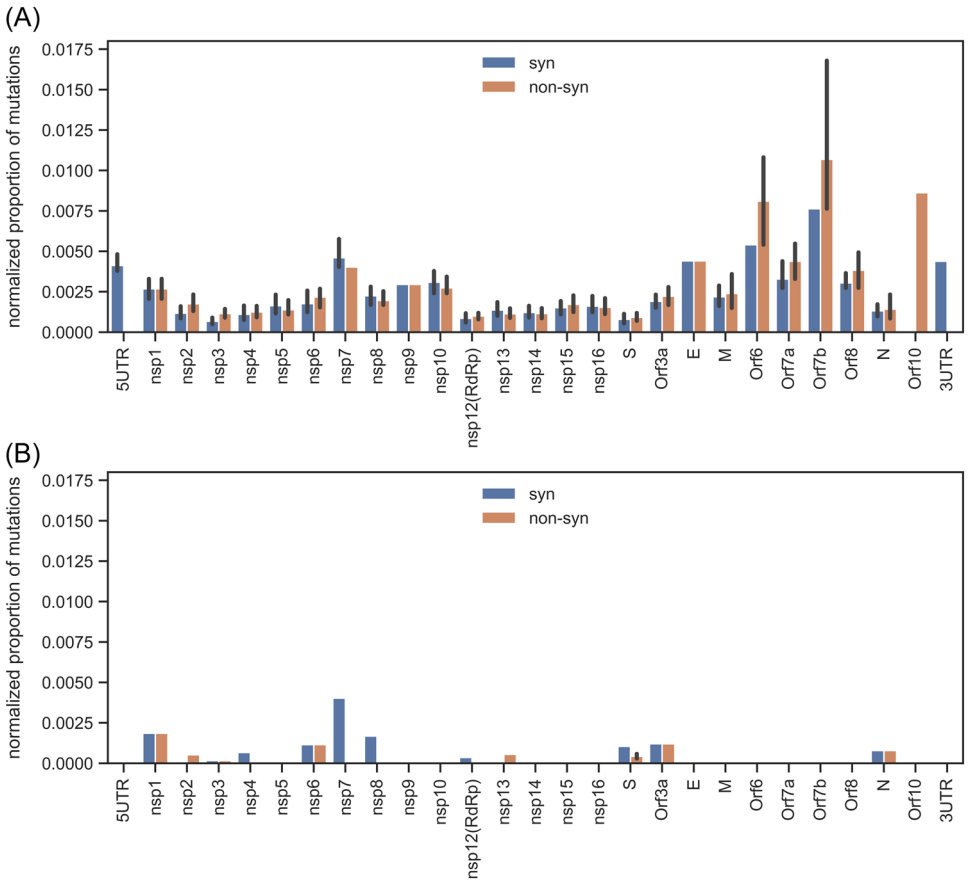

FIGURE 5 Synonymous versus nonsynonymous mutations within each gene of the SARS-CoV-2 genome after molnupiravir (A) and tixagevimab/cilgavimab (B) treatments. The value is normalized according to the base-pair length of each gene 5–95 percentile range is reported with black bars. Several genes are represented without bars because, for each of these genes, a single mutation was found in a single patient.

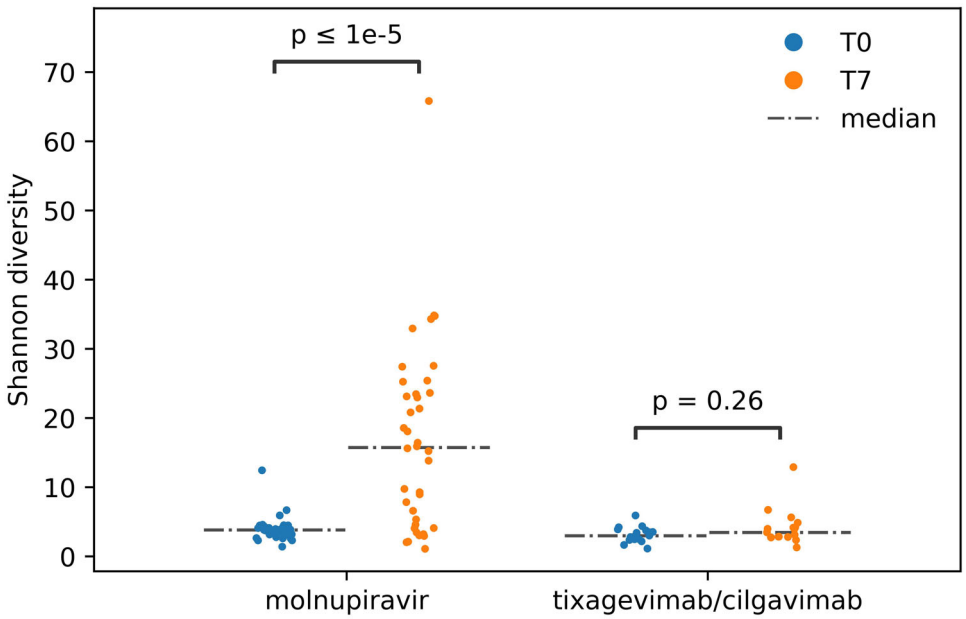

FIGURE 6 Shannon diversity after molnupiravir and tixagevimab/cilgavimab treatments.

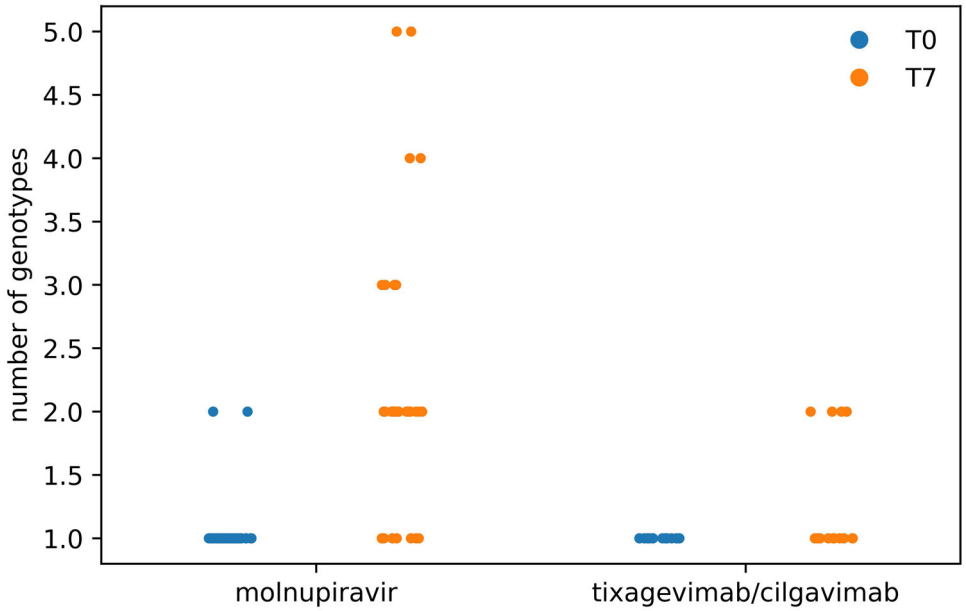

FIGURE 7 Quasispecies complexity after molnupiravir and tixagevimab/cilgavimab treatments.

we observed that molnupiravir treatment was able to induce an early complete virological response in many patients at Day 7, and among those who are still SARS-CoV-2 RNA positive after 7 days of treatment, most exhibited a significant drop in Ct values compared to the baseline. Notably, no difference between immunocompromised and immunocompetent patients has been observed.

To our knowledge, this is the largest study that attempts to correlate SARS-CoV-2 genetic variability with early treatment with molnupiravir. We found that molnupiravir administration rapidly increases the transition-to-transversion ratio, although this increase (9:1) appears to be lower than that reported in other studies (14:1).[4,14] In this regard, it should be noted that our study uses a different experimental approach. For SNV calculation, we considered all mutations (drug-related and drug-unrelated) also present in minoritarian SARS-CoV-2 sequences: this approach can be methodologically more appropriate, giving a picture of the total number of mutations in the viral population that is more complete than that obtained from the analysis of the consensus sequence alone. Furthermore, we ruled out any effect of APOBEC in inducing the appearance of transitions and demonstrated that the SNV distribution along the genome was uniform and did not preferentially affect proteins that represent the targets of the drug. This is in contrast with a recent study which identified select regions that were completely unaffected, leaving the authors to hypothesize that mutations in those regions likely abrogates infectivity.[30]

Importantly, in the control group of tixagevimab/cilgavimab patients, we observed values of the transition-to-transversion ratio very similar to those reported in the literature (3.6:1 vs. 2.66:1).[13,31,32] Moreover, in the present study, we observed a higher increase in the G->A mutation in molnupiravir-treated patients compared to the control group, as well as a higher total number of transitions (Figure 3). Such observation agrees with previously published works, where C->T and G->A mutations were defined as dominant in patients after molnupiravir treatment.[20,22]

In our study, we went further to ascertain the possible effect of molnupiravir treatment on the appearance of synonymous versus nonsynonymous mutations. When codon degeneracy was considered, G->A mutations led to nonsynonymous mutations in eight out of 12 cases, while C->T mutations led to nonsynonymous mutations in six out of 12 cases. Thus, considering that G->A and C->T represent our cohort's most detected types of mutations, we end up with 14 nonsynonymous mutations for every 24 mutations. The lack of any effect on the prevalence of synonymous versus nonsynonymous mutations was expected based on the molnupiravir mechanism of action.

Most importantly, we studied the effects of molnupiravir treatment on SARS-CoV-2 quasispecies to get a better understanding of intrahost evolution posttreatment. We limited the study of quasispecies composition to the spike gene of the SARS-CoV-2 genome, as this protein is the most important in the interaction between host and pathogen, and because viral evolution in the control group (treated with antispike antibodies) was expected to be limited to the spike gene only. Our findings document a pattern of changes to the quasispecies of the spike gene in a BA.5 omicron population, revealing key viral traits (increase in genomic complexity and diversity) induced by molnupiravir. The lack of therapy-driven changes in SARS-CoV-2 quasispecies after tixagevimab/cilgavimab treatment further supports the concept that molnupiravir can hardly impact SARS-CoV-2 diversity and complexity in treated patients. Our study has some limitations. The first limitation concerns the lack of a control group treated with other antiviral agents. However, other antiviral drugs act through mechanisms other than mutagenesis. Moreover, we cannot investigate the clinical significance at population level of such genetic variability, as for the transmissibility of this molnupiravir-mutated virus. However, the low Ct values observed at T7 in all sequenced samples is suggestive of an active replication in action, thus could result in

effective transmission of newly drug-induced possibly emerging variants. In conclusions, our data confirm the suspect that molnupiravir-generated variants can be generated also in immunocompetent outpatients[33] and highlight the importance of genomic surveillance in the characterization of new, potentially transmissible drug-derived variants.

AUTHOR CONTRIBUTIONS

Cesare Ernesto Maria Gruber, Emanuela Giombini, Martina Rueca, and Daniele Focosi conceived the study and developed the study protocol. Enrico Girardi and Fabrizio Maggi provided oversight and supervision. Martina Rueca, Lavinia Fabeni, Giulia Berno, Ornella Butera, Eliana Specchiarello, and Fabrizio Carletti did the molecular assays and sequencing. Valentina Mazzotta, Ilaria Mastrorosa, Silvia Rosati, Emanuele Nicastri, and Andrea Antinori collected the clinical data. Fabio Giovanni Tucci, Emanuela Giombini, and Cesare Ernesto Maria Gruber did the bioinformatic analyses of the data. Cesare Ernesto Maria Gruber, Emanuela Giombini, and Fabio Giovanni Tucci wrote the original draft of the manuscript. Fabrizio Maggi, Andrea Antinori, and Daniele Focosi critically reviewed the manuscript. All authors had access to the data in the study and were ultimately responsible for deciding to submit it for publication.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

All data generated in this study are available upon reasonable request to the corresponding author.

ETHICS STATEMENT

The study was conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee of the National Institute for Infectious Diseases INMI "L. Spallanzani" (protocol code 214 of 20/11/2020). Patient consent was waived due to the approval by the Ethics Committee of the National Institute for Infectious Diseases, INMI, "L. Spallanzani" (Comitato Etico INMI Lazzaro Spallanzani IRCCS/Comitato Etico Unico Nazionale COVID-19"; issue n. 214/20 November 2020) to use residual samples of COVID-19 patients for research purposes in anonymized form.

ORCID

Cesare Ernesto Maria Gruber http://orcid.org/0000-0002-8831-3380
Valentina Mazzotta http://orcid.org/0000-0002-0240-7504
Martina Rueca http://orcid.org/0000-0001-5162-046X
Daniele Focosi http://orcid.org/0000-0001-8811-195X

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