Cumulative SARS-CoV-2 mutations and corresponding changes in immunity in an immunocompromised patient indicate viral evolution within the host

Sissy Therese Sonnleitner1,2,6✉, Martina Prelog3,6, Stefanie Sonnleitner1, Eva Hinterbichler1, Hannah Halbfurter1, Dominik B. C. Kopecky1, Giovanni Almanzar3, Stephan Koblmüller4, Christian Sturmbauer4, Leonard Feist5, Ralf Horres5, Wilfried Posch2 & Gernot Walder1

Different scenarios explaining the emergence of novel variants of concern (VOC) of the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) have been reported, including their evolution in scarcely monitored populations, in animals as alternative hosts, or in immunocompromised individuals. Here we report SARS-CoV-2 immune escape mutations over a period of seven months in an immunocompromised patient with prolonged viral shedding. Signs of infection, viral shedding and mutation events are periodically analyzed using RT-PCR and next-generation sequencing based on naso-pharyngeal swabs, with the results complemented by immunological diagnostics to determine humoral and T cell immune responses. Throughout the infection course, 17 non-synonymous intra-host mutations are noted, with 15 (88.2%) having been previously described as prominent immune escape mutations (S:E484K, S:D950N, S:P681H, S:N501Y, S:del(9), N:S235F and S:H655Y) in VOCs. The high frequency of these non-synonymous mutations is consistent with multiple events of convergent evolution. Thus, our results suggest that specific mutations in the SARS-CoV-2 genome may represent positions with a fitness advantage, and may serve as targets in future vaccine and therapeutics development for COVID-19.
In December 2019 the Wuhan Municipal Health Commission (China) reported a cluster of cases of pneumonia of unknown etiology to the WHO China Country Office. By the beginning of 2020 it was confirmed that a novel coronavirus later named severe acute respiratory syndrome coronavirus (SARS-CoV-2), was the causative agent. SARS-CoV-2 spreads easily and effectively among human beings with a basic reproduction number (R0) of >2-3. Following this rapid human-to-human transmission and intercontinental spread the WHO declared a global pandemic in March of 2020. The first cases in Austria were reported in Ischgl, Tyrol, as early as February 2020—and East Tyrol was considered one of the first hotspot areas in Central Europe.

While mutations are common in RNA viruses and mostly will not make a significant difference, some mutations proved to provide SARS-CoV-2 with a selective advantage, such as increased transmissibility or increased escape from specific antibodies. Those variants with proven or suspected immune escape mutations were deemed variants of concern (VOC) or variants of interest (VOI), respectively, and require close monitoring. The spread of the first described variant of concern (Alpha variant, B.1.1.7, VOC) was confirmed early in Austria and increased from 0.7% in January to >99% in April 2021 in the study area of East Tyrol. Here, we describe the case of an immunocompromised patient with lymphoma who showed persistently high pharyngeal viral shedding for a period of more than four months. Two of the patients treated with monoclonal and convalescent plasma did not contain anti-SARS-CoV-1 or anti-SARS-CoV-2 antibodies according to the manufacturer and did not contain anti-SARS-CoV-1 or anti-SARS-CoV-2 antibodies according to the manufacturer’s quality checks (personal communication with the manufacturer). No antiviral therapeutics were administered to the patient at any time of the infection due to the relatively mild course of the infection according to the recommendations of the position paper of the Working Group of Scientific Medical Societies (AWMF) at this time point. A summary of the patient’s medical history is given in Table 1. An increase in leukocytes (21,600/µL) was detected in the final measurement at the end of the infection in June 2021. Finally in June 2021, after six months of persistent viral shedding, two doses of COVID-19-mRNA vaccine (BNT162b2; Comirnaty, BioNTech/Pfizer) were administered. Since the onset of symptoms and the first RT-PCR positive swab the patient was committed to home quarantine in accordance with Austrian law. When symptoms did not clear after a month, home quarantine was slightly lightened, but, testing with nasopharyngeal swabs and subsequent RT-PCR was continued. Persistent viral shedding was determined via qPCR at 25 time points across a 207-day-long period.

Results
Clinical presentation of an immunocompromised individual persistently infected with SARS-CoV-2. In August 2015, a female patient in her 60s was diagnosed with stage IVa small cell lymphocytic lymphoma, complicated by a temporary reactivation of Epstein-Barr virus (EBV) with reactive splenomegaly and rapid nodal progression. Beginning in June 2016, she was given six cycles of Rituximab and Bendamustine, which led to remission. In October 2019, the patient suffered a relapse with washout and 90% bone marrow infiltration (B-CLL Binet B or RAI III), accompanied by pronounced symptoms and antibody deficiency. Beginning in May 2020, another round of therapy with Rituximab and Bendamustine was administered. It was completed in November 2020 after six cycles. At that time, the leukocyte count was in the lower normal range at 4200/µL, platelets 136,000/µL, the immunoglobulins were clearly reduced (IgG 249 mg/dL, IgA 3 mg/dL, IgM 12 mg/dL).

Four days after the last chemotherapy—mid November 2020—the patient fell ill with fever, cough, headache and pain, but neither loss of taste nor smell. SARS-CoV-2 was detected in the throat swab by RT-PCR. The patient was in quarantine for 10 days; a final RT-PCR control was not carried out. Due to persistent fatigue, recurrent fever episodes and persistent cough with non-purulent secretion, the patient was again admitted to the hospital in the middle of January 2021 and RT-PCR was again positive for SARS-CoV-2. At the same time there was a recurrence of EBV. The patient was enrolled for an inhalation therapy with N-chlorotaurine (3 times daily inhalation of 10 mL of N-chlorotaurine for 3 min and 10 days; a very small percentage of the Gamma variant (P1 or B.1.1.238 and the spike deletions 144/145 described as recurrent escape effect can be found in several VOC or VOI. For example, N501Y in the Alpha, Beta and Omicron variant, a nucleotide substitution with lymphoma who showed persistently high pharyngeal viral shedding, the strain convergently developed a number of mutations which to a high degree have already been described in the context of variants of concern. The study shows the chronology of the evolution of intra-host mutations, which can be seen as the straight mutational response of the virus to specific antibodies and should therefore be given special attention in the rating of immune escape mutations of SARS-CoV-2. Our study reveals immunocompromised patients as a potentially new source of virus variants and therefore, emphasises the need to globally give this vulnerable group priority for vaccination.
The isolation success correlated negatively with the Ct-values of SARS-CoV-2 during the study period of 221 days is given in Table 3. Overall, 22 non-synonymous mutations accumulated intra-host and their concordance to VOC and VOI are given in Table 3. Eight of those were temporary. Seventeen of the 22 acquired non-synonymous mutations were persistent, whereas eleven (50%) occurred temporarily and were replaced by the wildtype or a different substitution. Seventeen of the 22 non-synonymous mutations evolved in the spike-coding region, eight of those were temporary. Seventeen of the 22 acquired non-synonymous mutations (77.3%) were issued as immune escape mutations by the WHO (https://www.who.int/en/activities/tracking-SARS-CoV-2-variants/) (Fig. 4). Among the persistent non-synonymous mutations in the spike, as many as 88.2% are found in various VOIs or VOCs. All of these changes occurred after the development of high antibody titres. One region continuously showed diffuse mutational changes, with temporary substitutions and deletions, which was ORF1b: position 709–716.

An overview of the intra-host mutational development of SARS-CoV-2 during the study period of 221 days is given in Table 4.

### Table 1 Summary of the patient’s demographic data and latest medical history.

| Age     | 60–70 |
|---------|-------|
| Gender  | Female |
| 2015 Aug | Diagnosis of IV a small cell lymphocytic lymphoma |
| 2016 June | Temporary reactivation of EBV |
| 2016 June | 6 cycles of Rituximab and Bendamustine |
| 2019 Oct | relapse |
| 2020 May | 6 cycle Rituximab and Bendamustine |
| 2020 Nov | End of the last cycle of chemotherapy |
| 2020 Nov | SARS-CoV-2 positive |
| 2021 Jan | Reactivation of EBV |
| 2021 Jan | IVIG therapy |
| 2021 June | BNT162b2, Comirnaty, BioNTech/ Pfizer, 2 doses at intervals of 3 weeks |

SARS-CoV-2 specific T-cell response. On day 193 no IFN-γ-producing SARS-CoV-2-specific immune cells could be detected in the ELISPOT assay (SI = 0.86), although a significant positive reaction against pokeweed mitogen was demonstrated (mean of 213 SFU in the positive control versus mean of 1.4 SFU in the negative control and mean 1.2 SFU cells in the SARS-CoV-2-antigen stimulated wells).

Humoral immune response did not clear SARS-CoV-2 infection. The Ct values and numbers of PFU/mL were significantly lower after day 124. The high titre of IgG antibodies of 1320 AU/mL and a neutralizing antibody titre of 1:32 analyzed by our in-house assay on day 124 was associated with a significant reduction of the viral load but could not clear the infection. We therefore decided to undertake a detailed examination of the specific genetic background of the virus population present including potential intra-host mutational dynamics.

### Mutational intra-host dynamics

Over the study period of 221 days, 14 haplotypes were sequenced out of naso-pharyngeal samples. The sequences were obtained on day 73, 93, 109, 129, 133, 136, 143, 158, 164, 171, 182, 192 and day 207 of the patient’s prolonged infection. The timeline of infection and a chronology of intra-host non-synonymous mutational events are given in Figs. 2 and 3.

The calculation of the pairwise mutation distances did not show higher intra-host evolutionary rates in contrast to overall evolutionary rates of about 8.9 × 10^−4 substitutions per year.16,17 The pairwise distance between day 73 and day 171 was 4.4 × 10^4 in 98 days, implying a nucleotide substitution rate of 7.5 × 10^−4.

All NGS sequences were shown to belong to the prevalent Pangolin lineage B.1.1 and the Nextstrain clade 20B.

We became aware of the prolonged viral shedding after about two months and started to regularly sequence the patient’s subsequent swabs as of day 73. A listing of all persistent and temporary non-synonymous mutations that the strain has accumulated intra-host and their concordance to VOC and VOI are given in Table 3. Overall, 22 non-synonymous mutations evolved over the study period of 221 days (seven months). Eleven (50%) of these non-synonymous mutations were persistent, whereas eleven (50%) occurred temporarily and were replaced by the wildtype or a different substitution. Seventeen of the 22 non-synonymous mutations evolved in the spike-coding region, eight of those were temporary. Seventeen of the 22 acquired non-synonymous mutations (77.3%) were issued as immune escape mutations by the WHO (https://www.who.int/en/activities/tracking-SARS-CoV-2-variants/) (Fig. 4). Among the persistent non-synonymous mutations in the spike, as many as 88.2% are found in various VOIs or VOCs. All of these changes occurred after the development of high antibody titres. One region continuously showed diffuse mutational changes, with temporary substitutions and deletions, which was ORF1b: position 709–716.

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### Chronology of acquired mutations.

In the underlying clinical case the substitutions emerged in the following chronological order:

S:Y144 emerged immediately after the increase of the specific antibody titre at day 117 as a temporary mutation, followed by E484Q (day 129), which could not assert itself against E484K and was displaced at least seven days later (day 136). Furthermore, we found the substitutions S:N354K (day 158, 164, 171 und 182), S:R346I (day 164) and ORF1a:T3284I (day 171), S:R950N (day 171) as well as the prominent S:K681H on day 182 (Fig. 2). Three of the six acquired substitutions (50%) have already been described as typical mutations acquired by diverse VOC.

Thirteen of the seventeen acquired substitutions (76.5%) occurred in the genomic spike-coding region, and one each in the regions coding for ORF1a:T3284, ORF3a:V255X (day 73), ORF8:Y73C (day 73) and N:S235F (day 136) (Fig. 2).

Other mutations appeared temporarily and were subsequently replaced by the wildtype variant. Five hitherto undescribed temporary mutations were observed on the days 73
Six temporary mutations have already been described previously, all of them prominent variations known in the context of VOC (https://covariants.org/shared-mutations; Center for Disease Control and Prevention; https://www.cdc.gov/coronavirus/2019-ncov/variants/variant-info.html): ORF8:Y73C and S:T716I evolved in the early stage of the infection and are described in the context of the Alpha variant, S:N501Y, known from the Alpha, Beta, Gamma and Omicron variant, S:del(9) described in the Beta variant, N:S235F, known as a typical substitution of the Alpha variant and S:H655Y, described in the context of the Beta as well.
as Omicron variants. All of these temporarily recurring mutational events did not establish themselves permanently but rather disappeared and/or were dominated again by the wildtype variant. Figure 2 shows the acquired und temporarily acquired mutations of the investigated strain in the spike-coding region and demonstrates the high concordance of the acquired mutations with described VOC, above all the Alpha and the Omicron variant (15/17; 88.2%) and represents the adaptations in the spike-coding region. Further mutations in the genome were ORF1a:T3284I (day 171), ORF8:Y73C (day 73), ORF3a:V255X (day 73), N:S235F (day 136), ORF1b:L714- (day 158). Thirteen of the 17 mutations (76.5%) acquired in the course of the prolonged infectious phase are already described mutations in VOC. Ten of the 17 spike mutations occur in a similar or identical way in the Omicron variant (58.8%). The non-synonymous mutations S:del143, S:del144, S:N501Y, S:H655Y and S:P681H were developed in identical form in the Omicron variant. Additional non-synonymous mutations occurred at the amino acid positions S:142, S:144, S:145, S:484 (twice) in the investigated strain as well as in the Omicron variant, which, however, led to different expressions (S:del142 instead of S:G142D, temporally both S:Y144H versus S:144del and S:Y145X versus S:145del as well as S:E484Q and S:E484K instead of S:E484A). Overall, 17 of the 22 mutations (77.3%) acquired by the investigated strain convergently evolved in other VOC, mainly in the Alpha and Omicron variants. In the spike-coding region, the proportion of acquired mutations identical to mutations of VOC is even higher—15 of the 17 mutations acquired there (88.2%) are found in other VOC. Overall, SARS-CoV-2 developed eleven persistent mutations during the study period of 140 days as well as eleven temporary mutational events. The chronology of intra-host mutational events is displayed in Figs. 3 and 4. An overview of the total number as well as a characterization of mutations accumulated by the investigated strain during the 7-month study period are shown below.

In the first swab sample, whole genome sequencing did not detect any spike mutations in the investigated strain compared to the reference genome. The first spike variants appeared as E484K on day 133 as a heterozygotic mutation in 41.3% of the targeted reads. On day 136 the proportion of E484K increased to 76% and, after more than seven days (day 143), the new variant dominated with 100%, but decreased to 76.8% again on day 158. On day 171 the spike variant P681H was observed for the first time with a proportion of 24% and dominated within a couple of weeks reaching 100% on day 182. Three of the six acquired substitutions (50%) are previously described substitutions of immune escape variants, namely: S:E484K, S:D950N and S:P681H.

The fluctuating occurrence of adaptive mutations. The emergence of adaptive mutations did not occur in a linear fashion, but rather fluctuating. Frequently, new mutations arose at a certain time point to be later replaced by the wildtype variant. As shown in Fig. 4, the mutation rate shows an oscillating course with peaks around day 125, increasing until day 182. Simultaneously, the viral load decreased continuously until the patient had several consecutive negative SARS-CoV-2 RT-PCR tests beginning on day 232 and is therefore considered to be cured from the SARS-CoV-2 infection.

Table 3 Listing of all persistent and temporary non-synonymous mutations that the strain has accumulated over the 7-month study period.

| Mutations                        | n total | VOC/VOI [%] | n in spike | VOC/VOI | % |
|----------------------------------|---------|-------------|------------|---------|---|
| Acquired non-synonymous mutations: | 22      | 17 (77.3)   | 17         | 15      | [88.2] |
| Persistent non-synonymous mutations: | 11      | 8 (72.7)    | 9          | 8       | [88.9] |
| Temporary non-synonymous mutations: | 11      | 9 (81.8)    | 8          | 7       | [87.5] |

n total, number of all non-synonymous intra-host acquired mutations; n in spike, number of all non-synonymous intra-host acquired mutations in the spike-coding region; VOC/VOI, number of mutations that are found in comparable expression in a variants of concern (VOC) or a variant of interest (VOI). Source data are provided as a Source Data file.
Fig. 4 Representation of all acquired mutations during prolonged infection. The acquired and temporarily acquired mutations of the investigated strain in the course of a seven-month-long infection in an immunocompromised person. Overall, 17 persistent or temporary spike mutations were evolved, whereas nine (52.9%) turned out to be temporary and were subsequently replaced by the wild-type variant. *Temporary mutations, S1 spike 1, S2 spike 2, hr heptad repeat, RBD receptor binding domain. The mutations marked in orange are also found in the Omicron variant (B.1.1.529) in similar or identical expression (10 out of 17), mutations marked in red are found in other VOC (3 of 17; 17.6%). All acquired mutations occurred in the regions ORF1a ($n=1$), ORF1b ($n=1$), ORF8 (1) and the spike ($n=17$). Thirteen of the 17 mutations (76.5%) acquired in the course of the prolonged infectious phase are already described mutations in VOC. Source data are provided as a Source Data file.

Table 4 Overview of (A) substitutions and (B) deletions in the SARS-CoV-2 genome over a 7-month study period in an immunocompromised patient.

| (A) | Polymorphic substitutions | Strain-specific sub. | Acquired sub. | Temporary mutations acquired and lost sub. |
|-----|--------------------------|---------------------|--------------|------------------------------------------|
| ORF1b:D708A | EL73F | S:E484K* (day 136) | ORF8:Y73C* (day 73) |
| ORF1b:K709X | N/R203K | S:N354K (day 158) | ORF8:Y73C* (day 73) |
| ORF1b:Y710X | N/G204R | S:R346I* (day 164) | ORF3a:V555X (day 73) |
| ORF1b:Y710L | ORF1a:L758V | ORF1a:T3284I (day 171) | S:A831V (day 117) |
| ORF1b:Y711D | ORF1a:P971S | S:D950N* (day 171) | S:Y144H (day 129) |
| ORF1b:Y711X | ORF1a:M3221I | S:P681H* (day 182) | S:E484Q (day 129) |
| ORF1b:R712X | ORF1a:V3976F | ORF1b:P314L* | S:H655Y* (day 158) |
| ORF1b:V711E | ORF1b:S598I | ORF1b:P1000L* | N:S235F* (day 136) |
| ORF1b:L714X | ORF1b:K1208- | ORF1b:H716- | ORF1b:A1204- |
| ORF1b:L714X | ORF1b:K1208- | ORF1b:H716- | ORF1b:A1204- |

| (B) | Polymorphic deletions | Strain-specific del. | Acquired del. |
|-----|---------------------|----------------------|--------------|
| ORF1b:K709- | ORF1a:A1204- | ORF1b:LT14-* (day 158) |
| ORF1b:Y710- | ORF1a:E1205- | S:L141-* (day 164) |
| ORF1b:Y711- | ORF1a:K1208- | S:G142- (day 164) |
| ORF1b:R712- | ORF1a:T3284I | S:V143- (day 164) |
| ORF1b:N713- | ORF1a:K1208- | S:Y144-* (day 171) |
| ORF1b:L714- | ORF1a:E1209- | ORF1b:L714- |
| ORF1b:Q715- | ORF1a:E1210- | ORF1b:L714- |

Columns 1A and B show genetic variations in ORF1b, a region with repeated changes between substitutions and deletions, i.e. polymorphic substitutions and deletions. Columns 2A and B show substitutions and deletions in comparison to the reference genome Wuhan (GenBank: MN908947.3, RefSeq: NC_045512.2), strain-specific, manifested since the beginning of the infection and maintained throughout the 7-month study period. Columns 3A and B show the chronology of all mutations acquired by SARS-CoV-2 during the intra-host evolutionary process. *Mutations of concern or mutations which are described in the context of immune escape. Column 4 shows temporary mutational events which occurred once and did not occur any more in the following sequence. sub., substitutions; del., deletions; α, mutations are described for the Alpha variant B.1.1.7; β, Beta variant B.1.351; γ, Delta variant B.1.617.2; δ, Omicron variants B.1.1.529. Underlined mutation sites are also found in the Omicron variant, but with a different substitution. Source data are provided as a Source Data file. The table lists all acquired substitutions (Table 4A) and all acquired deletions (Table 4B).
Intra-host evolutionary history. The intra-host evolution of the investigated strain from day 73 and the quasi-species arising from it in the course of the intra-host evolution form a distinct clade in the consensus tree and group together. The clade is placed among others comprised of strains and VOC found in Austria and uploaded to the GISAID platform in the same study period from January to May 2021 (Fig. 5). Early Austrian sequences of the Omicron variant from December 2021 were included subsequently.

Discussion
In this unique case report we described the dynamics of intra-host mutational events in an immunocompromised patient during a seven-months period of prolonged viral shedding and proven infectivity. We considered the possible influence of a quantitatively strong but regarding binding capacities probably functionally ineffective humoral antibody response and a lack of cellular immune response on the site-directed mutagenesis of SARS-CoV-2.

Our sequencing approach resulted in high-confidence variant identification and robust genome-wide coverage and enabled the establishment of a chronology of immune escape mutations. In addition, previously undescribed site-directed base-exchanges, found in the regions ORF1a and b (n = 2; ORF1a:T3284I, ORF1b:L714I), ORF3a (ORF3a:V255X) and spike protein (n = 2; SN354K, S:A831V), were described here. Four different well-established serological methods, namely CLIA SARS-CoV-2 TrimericS IgG, microarray immunoblots, neutralization test and Anti-IgG-SARS ELISA with the evaluation of the Anti-IgG-SARS-avidity gave insights into the humoral immune response and demonstrated the inability to clear the SARS-CoV-2 infection despite positive antibody responses. This may be due to the relatively low neutralizing ability of the detected IgG which is also supported by the low avidity of the specific IgG and impaired avidity maturation over time. Administration of IVIG was not able to enhance the clearance of SARS-CoV-2. Cellular immunity was diminished in this patient and the lack of adapted T cell-mediated immune defence may have contributed to the inefficient clearance. Indications for a reduced SARS-CoV-2 specific cellular immunity were given by the negative IFN-γ ELISpot. The substitution rate for SARS-CoV-2 was estimated as \(8.9 \times 10^{-4}\) nucleotides per site per year\(^{16}\). This is comparable to previously reported substitution rates of SARS-CoV (8.0–23.8\(^{10^{-4}}\))\(^{18}\) and MERS-CoV (11.2\(^{10^{-4}}\))\(^{19,20}\) and comparable to the reported substitution rates for Influenza A (4–5\(^{10^{-4}}\)) and Influenza B (2 \(\times 10^{-3}\)) virus in the haemagglutinine gene\(^{21}\). From this substitution rate it can be estimated that SARS-CoV-2 undergoes about one genetic change every other week\(^{16}\). In comparison, the nucleotide substitution rate per site and per year for Ebola virus (EBOV Makona) is estimated to be \(-1.2^{10^{-1}}\) and for HIV-1 (3.21–4.06)\(^{10^{-3}}\). Interestingly, the evolutionary rate stayed constant throughout the first months of infection and no significant change was measured after the increase of specific antibodies on day 124. We did not find elevated intra-host substitution rates compared to the general rate reported for SARS-CoV-2\(^{216}\). This unaltered intra-host evolutionary rate

![Fig. 5 Outgroup-routed consensus tree.](image-url)
compared to the global average evolutionary rate suggests that these mutations in an immunocompromised patient, driven by specific antibodies, do not lead to more frequent random genomic changes, but on the contrary to very specific targeted ones.

We assume that the presence of specific antibodies forced directional selection on retaining or regaining infectiousness and thereby strongly favored directional mutations at particular sites, acting as immune escape mutations.

E484K, a substitution in the receptor binding domain (RBD) appeared early in the course of the infection and is described to impair neutralization resistance34, potentially compromising vaccines effectiveness45–47,49. E484K is a well-established distinction in the VOC B.1.1.7 with E484K, P1, P2, B.1.315, B.1.525, B.1.526 as well as B.1.617.1 (Center for Disease Control and Prevention; https://www.cdc.gov/coronavirus/2019-ncov/variants/variant-info.html). At the same position E484, both subtypes of the Omicron variant have formed the alternative substitution alanine A (Center for Disease Control and Prevention; https://www.cdc.gov/coronavirus/2019-ncov/variants/variant-info.html). On day 158, the Omicron-specific mutation S:H655Y35,34 could also be detected as a temporary substitution. A further genomic change in the spike-coding region was identified on position S:N354K on day 158 and had never been described before. R346I was detected in the sequence of day 164. This mutation was previously described as a reaction of SARS-CoV-2 after monoclonal antibody treatment, seeming to maintain ACE2 binding activity35 and has also developed in the VOI Mu, 21H, B.1.621 (https://covarionvariants/variant-info.html). S:D950N arose around day 171 and is as adaptive mutation assigned to the Gamma and Delta variant (Center for Disease Control and Prevention; https://www.cdc.gov/coronavirus/2019-ncov/variants/variant-info.html).

The substitution P681H was observed for the first time in the sequence of day 171, whereby the amino acid histidine (H) first appeared as a polymorphism to become the dominant and finally fixed variant in the course of the next ten days. This transformation from proline (P) to histidine is relatively well studied and implicates a modification in the neighboring furin cleavage site at the junction of the spike protein receptor-binding (S1) and fusion (S2) domains36.

Another major transformation of the spike protein is the deletion of the amino acids 141 to 144. The deletions Y144/145 on the edge of the spike tip are modifications described in the VOC B.1.1.7 as the recurrent deletion region 2 (rd2), occurring repeatedly in SARS-CoV-2 variants 9,10.

In our case, the deletions were extended to three more deleted positions on S:141, 142 and 143. S:143del is another analogy to the Omicron variant B.1.1.592.

Nine persistent mutations were found in the spike-coding region. More precisely, four are located in the N-terminal domain (S:L141-, S:G142-, S:Y143-, S:Y144-), three in the RBD (S:R346I, S:N354K and S:E484K), both parts of S1 and three are positioned in the region encoding for S2, namely P681H in the immediate neighborhood of the furin cleavage site and S:D905N near heptad repeat 1 as part of the fusion core region.

Of the eleven acquired adaptive mutations, only two were found outside the spike-coding regions, namely L714- in ORF1b (day 158) and T3284I in ORF1a (day 171). ORF1a and ORF1b are coding regions for non-structural proteins (nsp)37. ORF1a:T3284I is located in the region encoding for nsp5. Nsp5 is regarded as the main protease, cleaves viral polypeptide and works closely with nsp12 and nsp13. Together, nsp5, 12 and 13 represent the replicase machinery.37–39. ORF1b:L714- is a deletion in the region coding for nsp13, the enzyme helicase, a main component of membrane-associated replication-transcription complexes 37,40–42.

It is remarkable that nine of the eleven persistent mutations (81.8%) acquired in the course of the prolonged infection had previously been described in the context of immune escape and were assigned to diverse VOC. Our bioinformatic analyses revealed that 75% of the novel mutations in our investigated strain also occur in VOC, whereas the highest concordance was found between the investigated strain and the Omicron variant (50%). Furthermore, we found dynamic mutational events with fluctuations between the wildtype and the variational mutation. Nine of these temporary mutations (9 of 11; 81.8%) have also been described in the context of VOC (Center for Disease Control and Prevention; https://www.cdc.gov/coronavirus/2019-ncov/variants/variant-info.html). In the close proximity of the acquired deletion in ORF1b:L714-, which manifested homogeneously, additional conspicuous polymorphic sequences in amino acid position ORF1b:708–716 were found. We measured frequent changes in substitutions and deletions, leaving us with the impression that the constant changes in the genetic structure display immune escape mutations. Also, this hotspot in directional mutations encodes for nsp13, the helicase.

We thus suggest that the accumulated mutations result from an increased selection pressure on the spike, the key to entering the host cell. At the same time a second process takes place intra-host, which exerts increased pressure and enforces continual reconstructions in the nsp13 region. The findings of these temporary mutations, which almost exclusively occurred in the spike region, also fit this pattern very well.

We managed to isolate SARS-CoV-2 from swabs at different time points, which is further evidence for the continuous viability of the virus over the study period, given the evolutionary dynamics of the different sequences. The isolation success correlated negatively with the Ct value; a fact that has already been observed in previous studies43.

Treatment with Rituximab resulting in depletion of particularly memory and effector B cells by targeting CD20 is known to cause impaired antibody responses44–46. As naïve B cell clones are less sensitive to Rituximab treatment due to their lower expression of CD20, a robust immune response can also be assumed for those patients. Immunosuppressive therapy as well as the lymphoma disease itself may have diminished the T cellular axis of immune defence against SARS-CoV-2, which targets infected cells, and loss of control by cytotoxic T cells may have caused the ongoing replication of SARS-CoV-2 in naso-pharyngeal epithelial cells47,48. Impaired T cell help may have contributed to the inefficient antibody maturation.

Meanwhile, there are more studies that shed light on the evolution of immune escape variants in immunocompromised patients and support the results of our study 12,49–55. Nonetheless, our study shows the accumulation of an unusually high number of immune escape mutations in a single patient, which to a strikingly high degree evolved in parallel in various VOC. The chronology of mutation events during seven months of infection shows a rapid accumulation of non-synonymous mutations which in part were persistent, in part temporary or even repeatedly acquired and lost.

In summary, our case report documents the medical phenomenon of persisting SARS-CoV-2 infection in an immunocompromised patient with impaired humoral and cellular immune response. Potential interference of specific antibodies led to a significant reduction in the viral load, but at the same time generated sophisticated escape mechanisms while the cell-mediated immune defence for eradication of the infection was missing. With the aid of NGS, we witnessed the directed mutational changes of SARS-CoV-2, probably facilitated by insufficient humoral immune defence. This led to the formation of highly specific virus variants, highlighting the regions exposed to the
highest intra-host selective pressure. Based on this observation one may hypothesize that immunocompromised patients pose a particular risk to accumulate immune escape mutations and hence be a source for new VOC. This clearly represents an additional risk factor to be considered in the future. Our study also underlines the importance to protect immunocompromised patients from SARS-CoV-2 infection by modified vaccination strategies. Most importantly, the study points out the convergent intra-host evolution of specific mutations in SARS-CoV-2, as they emerged independently in previously described VOCs, VOIs and in the strain we studied. Those specific, convergently evolving mutations reveal those neutralic positions in the SARS-CoV-2 genome that on the one hand represent its highest fitness advantage, but on the other hand also uncovers its highest vulnerability and should be considered as the probably most important points of attack in future vaccine and therapeutics development.

### Methods

**Immunological diagnostics.** CLIA SARS-CoV-2 Trimeric S IgG. Serological tests were performed using the LIAISON SARS-CoV-2 TrimericS IgG (DiaSorin S.p.A., Saluggia, Italy) (LIAISON), an Immunoblot called ViraChip assay (Viramed, Munich, Germany) and an in-house enzyme-linked neutralization assay (ELNA)56 at day 102, 124, 182 and 205 after the first positive PCR.

The LIAISON SARS-CoV-2 TrimericS IgG is a CLIA (Chemiluminescent Immunoassay) which detects IgG antibodies reactive with the spike protein (S1/S2 domain). The assay was performed on the LIAISON XL Analyzer according to the manufacturer’s instructions and gives the arbitrary units per ml (AU/mL) according to the WHO International Standards for the Anti-SARS-CoV-2-immunoglobulin-binding activity (NIBSC 20-136).

**Microarray immunoblots.** The ViraChip assay detects temporal antibody profiles of different immunoglobulin classes against S1, S2, and nucleocapsid (N) as well as against the spike domain (S1/S2). (Autoimmun Diagnostika, GmbH, Germany) for the four non-SARS human coronaviruses 229E, HKU1, NL63 and OC43 as a control of possible cellular cross-reactive responses. After incubation at 37 °C for 20 h in a sterile and humidified atmosphere, plates were washed with washing buffer (Autoimmun Diagnostika GmbH, Germany) for the four non-SARS human coronaviruses 229E, HKU1, NL63 and OC43 and then stained with the immunoglobulin-binding activity (NIBSC 20-136). The assay was performed on the LIAISON XL Analyzer according to the manufacturer’s instructions and gives the arbitrary units per ml (AU/mL). The cut-off titre for the PRNT, 1:4 in Medium199 containing 3% fetal calf serum. Equal volumes of virus (1 × 105 TCID50) and serum dilutions in Medium199 were mixed and subsequently incubated in a 5% CO2–humidified atmosphere, plates were washed with washing buffer (Autoimmun Diagnostika GmbH, Germany) for the four non-SARS human coronaviruses 229E, HKU1, NL63 and OC43 as a control of possible cellular cross-reactive responses. After incubation at 37 °C for 20 h in a sterile and humidified atmosphere, plates were washed with washing buffer (Autoimmun Diagnostika GmbH, Germany) for the four non-SARS human coronaviruses 229E, HKU1, NL63 and OC43 as a control of possible cellular cross-reactive responses.

**Neutralization test.** Neutralization ability of antibodies was determined performing an in-house enzyme-linked neutralization assay (ELNA)56,73. VeroB4 cells (ACC-33, DSMZ) were seeded in flat-bottom 96 well plates (Sarstedt, Germany) with Medium199 supplemented with 2% fetal calf serum (Thermo Scientific Gibco, USA) at a density of about 105 cells/ml to give a confluent monolayer. Next day, an infectivity titration was carried out to determine 100 tissue culture infectious dose 50% (100 TCID50).55,57 Sera were heat inactivated by incubation at 56 °C for 30 min. All sera were primarily assessed via a classical plaque reduction neutralization test (PRNT). To evaluate the cut-off titre for the PRNT, 10 sera of healthy East Tyrolean blood donors from the pre-pandemic years 2012 and 2013 were assessed in SARS-CoV-2 specific PRNT and ELNA. The cut-off titres were set at 1:32 with a viral solution of 100 TCID50 for PRNT and 1:4 with a viral solution of 1 × 105 TCID50 for ELNA. With these evaluated sera, we adapted the PRNT to an ELNA without the need of an apparent cytopathic effect (CPE) and a shorter incubation period of <24 h. For ELNA, sera were titrated in duplicate in twofold dilution steps, starting at a dilution of 1:4 in Medium199 containing 3% fetal calf serum. Equal volumes of virus (1 × 105 TCID50) and serum dilutions in Medium199 were mixed and subsequently incubated for 1 h at 37 °C. After addition of Medium199 (Thermo Scientific Gibco, USA) at a density of about 106 cells/ml to give a confluent monolayer. Next day, an infectivity titration was carried out to determine 100 tissue culture infectious dose 50% (100 TCID50).55,57 Sera were heat inactivated by incubation at 56 °C for 30 min. All sera were primarily assessed via a classical plaque reduction neutralization test (PRNT). To evaluate the cut-off titre for the PRNT, 10 sera of healthy East Tyrolean blood donors from the pre-pandemic years 2012 and 2013 were assessed in SARS-CoV-2 specific PRNT and ELNA. The cut-off titres were set at 1:32 with a viral solution of 100 TCID50 for PRNT and 1:4 with a viral solution of 1 × 105 TCID50 for ELNA.

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RT-PCR. RT-PCR extracts were evaluated for SARS-CoV-2 by qRT-PCR using the Bio-Rad CFX96 system (Bio-Rad, Germany) with a LightMix Modular Assay kit in accordance with the modified Chariti guidelines. 10 µl of extracted RNA were added into 15 µl 4x Reliance One-Step Multiplex Supernox (Bio-Rad, Germany). Each 15 µl mastermix contained 12.5 µl buffer solution, 0.25 µl enzyme mix, 1.75 µl of nuclease-free water and 0.5 µl primer probe hCoV-E (E-Gene, as well as N-Gene and RdRp- Gene for confirmation), 10 µl MoBiol, Cat. Nos. 53-0776-06 for 53-0775-96 and 53-0777-96. Reactions were incubated at 55 °C for 5 min and 95 °C for 5 min in order to conduct reverse transcription of viral RNA, sample denaturation and enzyme activation. These steps were followed by PCR-amplification including 45 cycles at 95 °C for 5 s, 60 °C for 15 s and 72 s for 15 s. Cooling was implemented at 80 °C for 30 s. Reactions were interpreted based on the Second Derivative Maximum (SDM) method. Positive results were confirmed by Rdp and N-gene samples with an initial Ct value lower than or equal to 37 were assigned to repeated testing including extraction. A Ct value higher than 40 was considered negative. Quantification of the viral load in the swabs was calculated via size standards of 1, 10, 100 and 1000 plaque-forming units (PFU)/mL. Standardization of viral stocks was carried out by virus titration. Isolation was performed on VeroB4 cells as described elsewhere.

Whole genome sequencing and mutational analysis. Libraries were prepared according to the Ion AmpliSeq SARS-CoV-2 Research Panel (Thermofisher, USA), library construction and sequencing protocol with the Library Kit Plus (Thermo Fisher Scientific, Waltham, Massachusetts, USA; Cat. No. 4488990). The AmpliSeq library was cleaned up with Ampure XP beads (Beckman Coulter, Germany) with a 1:1 ratio. The libraries were cleaned up using the Ion Library TagMan Quantitation Kit (Cat. No. 4468802), normalizing, pooling, and sequencing was performed using an Ion Torrent S5 Plus Ion Torrent Suite software (v 5.12.2) of the Ion Torrent S5 Plus Ion. Libraries were sequenced on the Ion Torrent S5 Plus Ion Torrent Suite software (v 5.12.2) of the Ion Torrent S5 Plus Ion. The generated full-genome sequences are available at Genome Sequence Archive as.bam files under the bioproject name PRJCA008966 (https://ngdc.cnbc.ac.cn/pa/browse/CRA006527). The sequences are deposited and available under the following accession numbers: CR453213 (day 73), CR453214 (day 93), CRR453215 (day 117), CR453216 (day 123), CR453217 (day 129), CR453218 (day 136), CRR453219 (day 143), CR453220 (day 150), CR453221 (day 164), CR453222 (day 171), CR453223 (day 182). Source data are provided with this paper.

Virus titration. Confluent VeroB4 cells were cultured in Medium199 including 5% FCS in T75 tissue culture flasks (Sarstedt, Germany) and transferred into 96-well tissue culture plates (Sarstedt, Germany). Passage 1 isolates of SARS-CoV-2 were thawed from –80 °C freezer and titrated from 1:10 to 1:10⁻¹² U-shaped 96-well plates (Greiner, Germany) and pipetted into each corresponding well of the 96-well tissue culture plate. Plates were incubated at 37 °C. Three days post infection, incubation was stopped by gently removing the supernatant, washing the cells three times with PBS and fixing cells in 1:1 ice-cold acetone–methanol. For easier optical evaluation, cells were dyed by crystal violet staining and tissue culture infectious dose 70% (TCID₅₀) and PFU were calculated.

Data availability. The generated whole genome sequences of days 73, 93, 117, 123, 129, 148, 164, 171 and 182 are available in the Genome Sequence Archive as.bam files under the bioproject name PRJCA008966 (https://ngdc.cnbc.ac.cn/pa/browse/CRA006527). The sequences are deposited and available under the following accession numbers: CR453213 (day 73), CR453214 (day 93), CRR453215 (day 117), CR453216 (day 123), CR453217 (day 129), CR453218 (day 136), CRR453219 (day 143), CR453220 (day 150), CR453221 (day 164), CR453222 (day 171), CR453223 (day 182). Source data are provided with this paper.

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Bioinformatics CoV-Seq Workflow. The frequency of the various mutations and the homology to the most widespread VOC (Alpha-, Beta, Gamma- and Delta-variant) were investigated based on BAM files. Reads from CoV-Seq samples were demultiplexed by using in-house tools. Reads originating from human were filtered out by mapping against hg19 with bwa-mem 0.7.17. All reads not mapping to human were trimmed for adapters und quality by using Cutadapt 3.27. The trimmed reads were mapped with bwa-mem 0.7.17 to the SARS-CoV-2 reference MN908947.3 from the NCBI. Mutations were called using breesq 0.35.57. Graphics were created using pandas 1.2 for Python 3.

Statistics. Dichotomous data were evaluated by a chi-squared test or Fisher’s exacta in the case of small group size (n < 60) (Microsoft Excel, Microsoft 395 MOSO, Windows 10). A two-sided significance level of p < 0.05 was used for determining statistical significance. After testing for distribution (Kolmogorov–Smirnov-test), non-parametric continuous independent variables were compared using Mann–Whitney–U-test for each time point. Dependent non-parametric variables were compared using Wilcoxon-rank test.
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Author contributions
S.T.S.: Conceptualization, data curation, formal analysis, funding acquisition, investigation, methodology; writing—original draft preparation. M.P.: Interpretation and discussion of clinical data and laboratory results, writing—original draft preparation, editing; S.S.: Methodology, writing—editing. E.H.: Investigation; methodology; H.H.: Project administration. D.B.C.K.: Visualization, organization. G.A.: Methodology. S.K.: Conceptualization, writing—editing, funding acquisition. C.S.: Conceptualization, writing—editing, funding acquisition. L.F.: Validation, verification. R.H.: Validation, verification. W.P.: Validation, supervision. G.W.: Supervision.

Competing interests
The authors declare no competing interests.

Additional information
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Correspondence and requests for materials should be addressed to Sissy Therese Sonnleitner.

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