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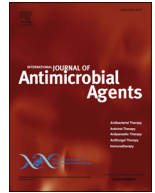


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Short Communication

Zanamivir and baloxavir combination to cure persistent influenza and coronavirus infections after hematopoietic stem cell transplant

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ABSTRACT

Objectives: Immunocompromised patients may experience prolonged shedding of influenza virus potentially leading to severe infections. Alternatives to monotherapy with neuraminidase inhibitors should be evaluated to entirely suppress viral replication and prevent drug-resistant mutations.

Methods: We investigated the clinical and virological evolution in a case of persistent influenza A and human coronavirus OC43 (HCoV-OC43) coinfection in a hematopoietic stem cell transplant recipient after different therapeutic strategies.

Results: Successive oseltamivir and zanamivir monotherapies failed to control both infections, with positive results persisting for over 110 days each. This led to the emergence of highly resistant oseltamivir strains due to neuraminidase mutations (E119V and R292K) followed by a deletion (del245-248), while maintaining sensitivity to zanamivir. The intra-host viral diversity data showed that the treatments impacted viral diversity of influenza virus, but not of HCoV-OC43. Considering the patient's underlying condition and the impact of prolonged viral shedding on pulmonary function, eradicating the influenza virus was necessary. A 10-day regimen combining zanamivir and baloxavir-marboxil effectively controlled influenza virus replication and was associated with the clearance of HCoV-OC43, finally resulting in comprehensive respiratory recovery.

Conclusion: These observations underscore the importance of further investigating combination treatments as the primary approach to achieve influenza eradication in immunocompromised patients.

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1. Introduction

Immunocompromised patients may experience prolonged shedding of respiratory viruses, which can result in severe infections [1] and may contribute to the emergence of variants with altered

transmissibility or immune characteristics. The intrinsic antiviral efficacy of neuraminidase inhibitors (NAIs), when used as a sole treatment in immunocompromised patients, may be insufficient to fully suppress influenza virus replication, which can lead to the emergence of treatment-resistant mutations [2,3]. The existence of different classes of anti-influenza molecules now allows considering drug combinations, which might present synergism, thereby enhancing the chance of success and reducing the risk of drug resistance emergence [4]. Herein, we present the case of a 37-year-old woman, who underwent an allogeneic hematopoietic stem cell transplantation (alloHSCT) and experienced a prolonged influenza

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A and human coronavirus (HCoV-OC43) coinfection that was not successfully treated with two consecutive monotherapies of NAIs. A combination of a NAI and a polymerase complex inhibitor proved effective in curing the infection.

2. Methods

2.1. Virus detection and follow-up

Respiratory viral infections were tested with a multiplex PCR assay (BioFire® Respiratory Panel 2.1 plus, bioMérieux, Marcy-l'Étoile, France). The levels of influenza A levels were estimated with cycle threshold values of specific real-time PCR assays (Xpert® Xpress CoV-2/Flu/RSV, Cepheid, VILLE and NeuMoDx™ Flu A-B/RSV/SARS-CoV-2 Vantage Assay, Qiagen, VILLE). Overall, 28 samples were collected (27 nasopharyngeal swabs and 1 saliva).

2.2. Sequencing

For metatranscriptomic sequencing, RNA was extracted using the QIAamp Viral RNA kit (Qiagen) and treated with Turbo DNase (Ambion). After ribosomal RNA depletion [5], RNA was reverse transcribed into double stranded cDNA using random hexamers, and libraries prepared using the Nextera XT kit (Illumina). Libraries were sequenced on an Illumina NextSeq500 (2 × 75 cycles).

Influenza virus genomes were also amplified from positive samples using an amplicon approach [6]. Libraries were prepared at the Mutualized Platform for Microbiology (P2M) using the Nextera XT kit (Illumina) and sequenced on an Illumina NextSeq500 (2 × 150 cycles). Sequencing statistics are presented in Supplementary Table 1.

Raw sequence data (human reads removed) were deposited in the European Nucleotide Archive (ENA) portal (<https://www.ebi.ac.uk/ena/>) under bioproject accession number PRJEB64657.

2.3. Genomes reconstruction

Adapters and low-quality sequences of raw reads were removed using Trimmomatic v0.39 [7]. Trimmed reads were assembled using megahit v1.2.9 [8] and contigs were queried against the NCBI non-redundant protein database using DIAMOND v2.0.4 [9]. HCoV-OC43 and influenza A H3N2 viruses were identified, with no other viral contaminants. Trimmed reads were then mapped on the scaffold genomes of each virus genome. The mapping data was visually checked to confirm the accuracy of the obtained genomes using Geneious Prime 2023. The novel HCoV-OC43 genome sequence from the earliest sample was deposited in GenBank (accession number OR371530), and the A/H3N2 genomes were deposited on GISAID (EPI_ISL_17512135, EPI_ISL_17512136, EPI_ISL_17512162 and EPI_ISL_17975828).

2.4. Intra-host viral diversity

Nucleotide diversity analysis was performed using a Python script (https://github.com/SimonLoriereLab/HCV_resistance) designed to process Variant Call Format (VCF) data obtained from iVar. We also computed the number of genomic positions with changes (minimum threshold of 3%, minimum read depth of 1000 reads), and their respective frequencies. For each virus, sequencing reads were down sampled to the level of the sample with the lowest amount of read mapped.

2.5. Phylogenetic and recombination/reassortment analysis

All available complete HCoV-OC43 genome sequence data and metadata were retrieved from BV-BRC [10] (<https://www.bv-brc.org/>) in May 2023, and a subset of genomes representative of described genotypes was selected [11]. Sequences were aligned together with the genome reconstructed from the earliest sample using MAFFT (v.7.467) [12] and the alignment was checked for accuracy using Geneious prime. We used a combination of six methods implemented in RDP4 [13](RDP, GENECONV, MaxChi, Bootscan, SisScan and 3SEQ) to detect potential recombination events in the newly reported genome. ModelFinder [14], as implemented in IQ-TREE v2.0.6 [15] was used to select the best-fitting nucleotide substitution model to infer maximum-likelihood phylogenies. Branch support was calculated using ultrafast bootstrap approximation with 1000 replicates [16]. The phylogenies were visualized using the auspice module from Nextstrain [17]. Interactive phylogenies are available at: <https://github.com/Simon-LoriereLab/OC43>. Phylogenetic analysis of the longitudinal genomes revealed that the patient samples all clustered together regardless of sampling date, indicative of a unique, persistent infection.

Similarly, a representative dataset of influenza A/H3N2 viruses was selected using the Nextstrain software subsampling module, with a grouping by year-month-country, from complete genomes data retrieved from GISAID (<https://gisaid.org/>). Sampling of individuals from the resulting selection ranged from 2017 to 2023. Alignment was performed with the “augur align” function of Nextstrain. We inferred the H3N2 phylogeny using IQ-TREE v2.0.6, using ultrafast bootstrap approximation with 1000 replicates to test for robustness. Potential reassortment events were investigated by comparing phylogenetic trees across genomic segments. Phylogenetic analysis of the longitudinal genomes revealed that the patient samples all clustered together regardless of sampling date, indicative of a unique, persistent infection.

2.6. Virus isolation and antiviral susceptibility

To attempt isolation of influenza virus, 100 µl of samples were inoculated in duplicate in 12-well plates containing Madin-Darby Canine Kidney MDCK (ATCC® CCL-34) cells, grown in Modified Eagle's Medium (MEM) supplemented with 5% FBS. After 1 hour adsorption at 35°C, 2 mL of MEM without FBS supplemented with 1% penicillin-streptomycin, 1% Fungizone and 1 µg/ml TPCK-treated trypsin was added. Cell morphology and the development of cytopathic effects (CPE) were observed daily for up to 7 days post infection and culture supernatant collected at Day 6 and 3 days after signs of CPE were observed. Virus titers were measured using a hemagglutination assay, and viral stocks characterized by metatranscriptomic sequencing.

2.6. Virus isolation and antiviral susceptibility

Oseltamivir susceptibility was measured using the NA-Fluor™ Influenza Neuraminidase Assay kit (Applied Biosystems). The signal was read on an Infinite M2000 PRO device (Tecan).

3. Results

3. Results

3.1. Case presentation

The patient was diagnosed with follicular lymphoma in 2017 and received bendamustine and rituximab treatment. Upon lymphoma relapse two years later, she received rituximab as monotherapy. The disease was controlled for a few months but subsequently progressed, and the patient received CAR-T cells therapy in 2020. In Spring 2022, she developed pancytopenia without evidence of relapse, which was classified as aplastic anaemia. Due to persistent pancytopenia despite eltrombopag treatment, she received an allogeneic bone marrow graft from an HLA matched unrelated donor in October 2022. The patient did not experience graft-versus-host disease. Hematologic recovery was late and incomplete, with granulocyte-colony-

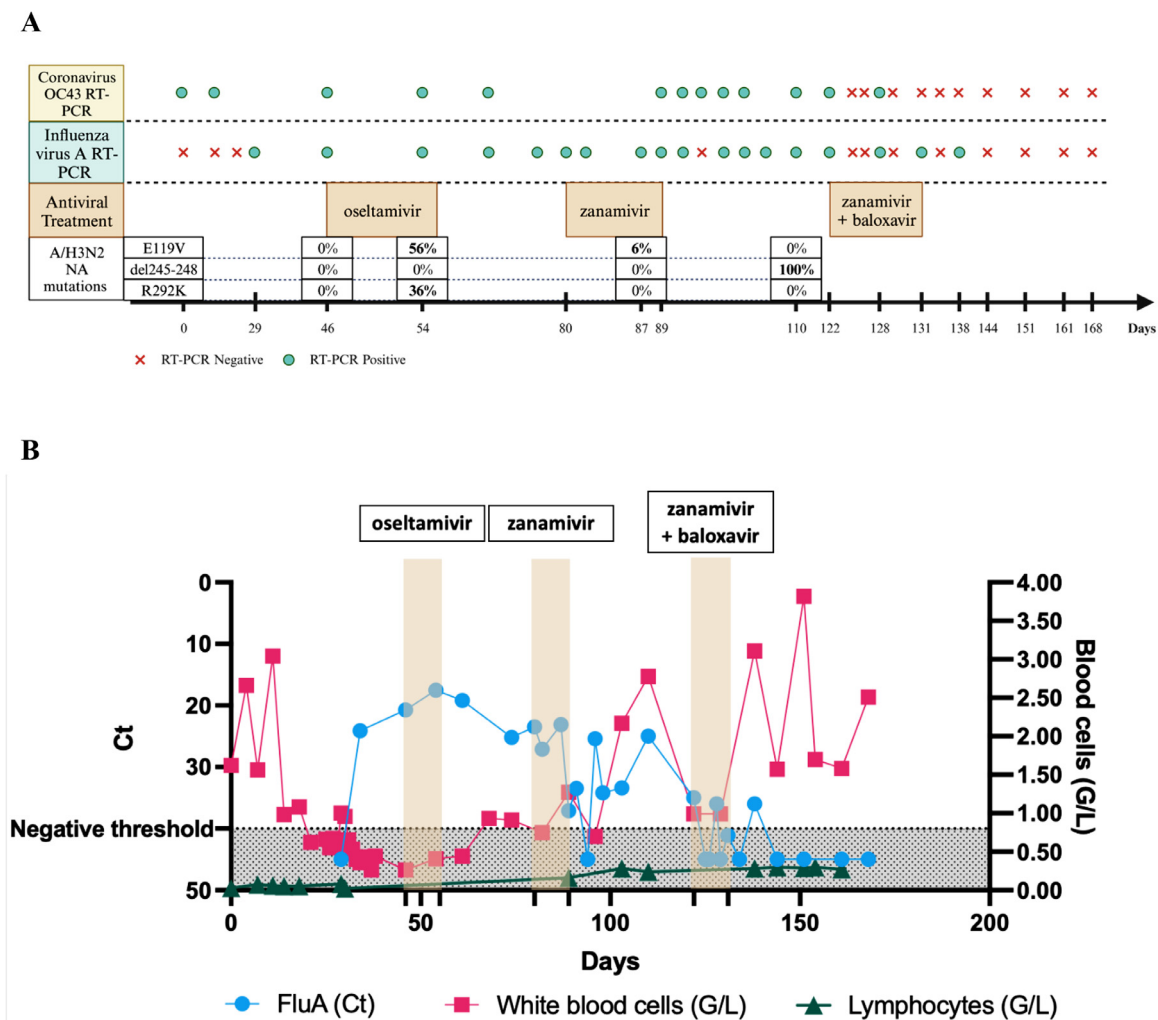


Fig. 1. Virus detection, antiviral treatments and neuraminidase mutations. Panel A. Timeline of virus detection, antiviral drugs administration and neuraminidase sequencing results. HCoV-OC43 and Influenza were detected using the BioFire® Respiratory Panel 2.1 plus (bioMerieux, Marcy-l'Étoile, France). Positive results are indicated by cyan dots, negative results are marked with red crosses. Anti-viral treatments are represented by yellow squares. Mutations in the neuraminidase (A/H3N2 NA) and their frequencies are noted in white squares. Panel B. Monitoring influenza infection and blood cell counts. Influenza A levels (blue circles) plotted on the left y-axis were estimated using cycle threshold (Ct) values from specific real-time PCR assays (Xpert® Xpress CoV-2/Flu/RSV, Cepheid, Sunnyvale, CA). The negative threshold value of 40 is represented by the dotted line. White blood cells (pink squares) and lymphocytes (green triangles), expressed in billions of cells per litre in whole blood (G/L), are plotted on the right y-axis. Influenza treatment timing is illustrated by yellow bars and corresponding molecules are indicated above. Day 0 corresponds to the date when HCoV- OC43 was first detected.

stimulating factor dependency and transfusion-dependent anaemia and thrombocytopenia. Chimerism was mixed, around 5% recipient in peripheral blood, and immunosuppressive treatment with cyclosporine was continued. Cytopenia persisted despite romiplostim treatment. The patient received the usual prophylaxis after alloHSCT, including amoxicillin, valacyclovir, cotrimoxazole, letermovir as well as posaconazole due to persistent neutropenia. After alloHSCT, she had not been vaccinated against influenza.

Two months after alloHSCT, the patient presented with a sore throat without fever or respiratory symptoms. A nasopharyngeal swab tested positive (day 0 [D0]) for HCoV-OC43 (Fig. 1), belonging to the newly proposed genotype J (Supplementary Fig. 1). The patient developed a cough, which persisted for four weeks. On Day 25, a nasopharyngeal swab was still positive for HCoV-OC43 only. Nine days later (D34), a subsequent nasopharyngeal swab identified the presence of influenza A virus (H3N2), belonging to the currently circulating A/H3N2 3C.2a1b.2a.2b clade (Supplementary Fig. 2).

3.2. Clinical course, anti-viral treatments

On D34 the patient had persistent cough without dyspnea, and normal auscultation, as well as oxygen saturation. On D44, symptoms persisted, and a nasopharyngeal swab was still positive for influenza A and HCoV-OC43. A computed tomography (CT) scan showed bilateral (predominantly left) bronchopneumonia of the bases. A 10-day course of oseltamivir 75 mg twice a day was started on D46 but was unable to control the infection (Fig. 1).

In the context of pancytopenia, hypogammaglobulinemia (4 g/L) and respiratory viral infection, monthly intra-venous immunoglobulin supplementation was started.

On D64, pulmonary function tests (PFTs) showed a moderate non-reversible obstructive respiratory disorder, prompting the initiation of inhaled steroids. A D74 CT scan showed no progressive thoracic anomalies but indicated pan-sinus filling. A new chest CT scan performed on D126, due to increased coughing with purulent morning sputum, revealed diffuse bronchial thickening, and D133-follow-up sinus CT scan showed stable pan-sinusitis.

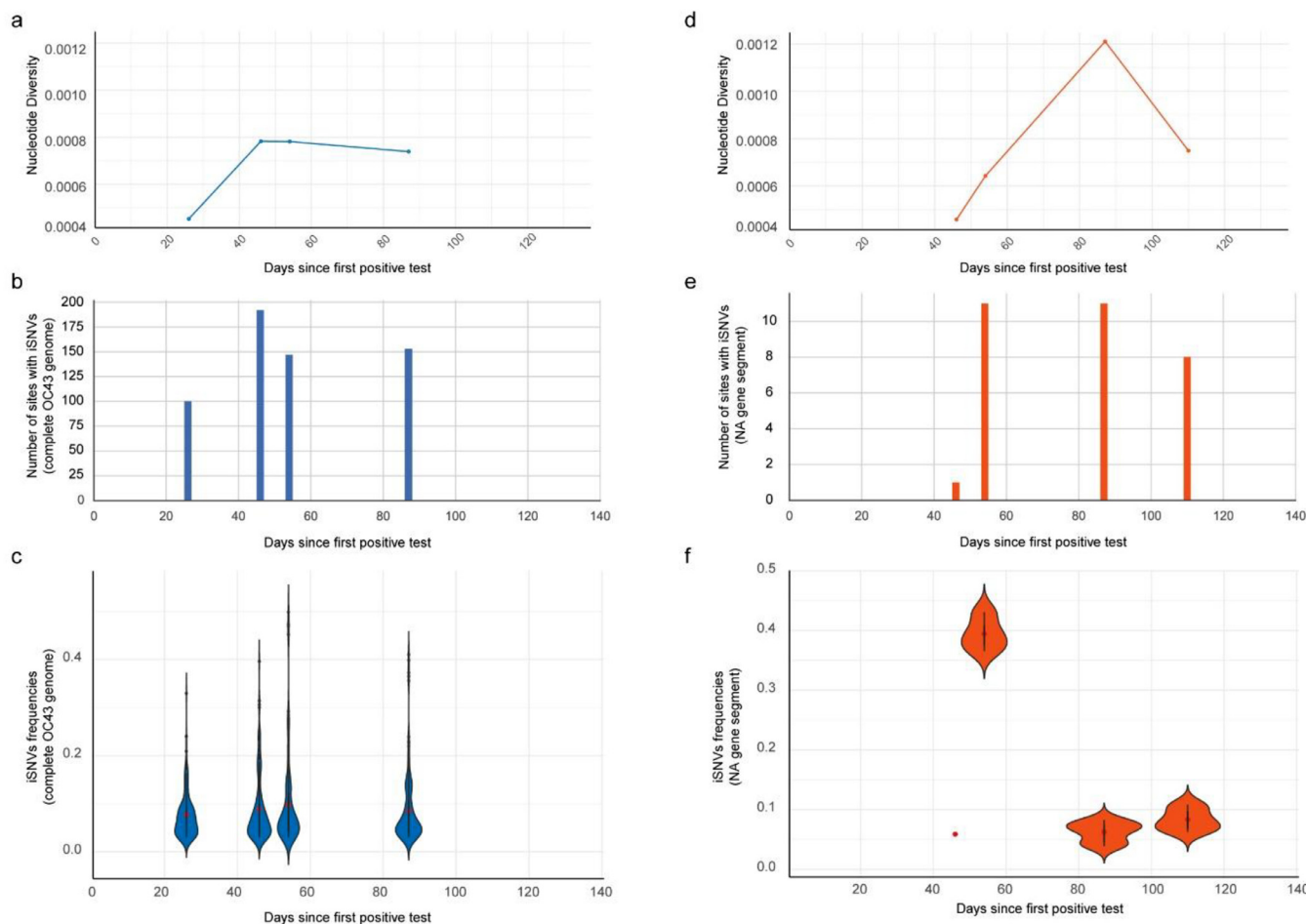


Fig. 2. Evolution of intra-host viral diversity. Diversity index estimated from the intra-host single nucleotide variations (iSNVs) data for the complete genome of OC43 (A) and the influenza H3N2 NA gene segment (D) for the respective snapshots captured over time. Number of sites with were detected iSNVs along the complete genome of OC43 (B) and the influenza H3N2 NA gene segment (E) for the respective snapshots captured over time. Violin plots representing the distribution of iSNVs frequencies the complete genome of OC43 (B) and the influenza H3N2 NA gene segment (E) for the respective snapshots captured over time.

During the patient's course, two 10-day courses (from D68 and D129) empiric piperacillin-tazobactam were administered for febrile episodes. Despite extensive work-up, no bacterial or fungal infections were identified. Weekly nasopharyngeal swabs were positive for influenza A and HCoV-OC43 for 104 days (Fig. 1).

After oseltamivir failure, the patient received a 10-day course of intravenous zanamivir at a daily dose of 600 mg, starting from D80. However, this treatment did not lead to either clinical improvement or viral clearance, and the viral load increased again after treatment withdrawal (Fig. 1A and B).

3.3. Virus variability and virus susceptibility to antivirals

Phylogenetic analyses were performed to characterize viruses (Supplementary Figs. S1 and S2). The HCoV-OC43 strain branched with genotype J (Supplementary Fig. S1). Recombination has been shown to have played a major role in coronaviruses evolutionary history [18] including impacting HCoV-OC43 genomic diversity [19]. The genome sequenced here did not present a signal of recombination within genotype J. Phylogenetic analysis of HA gene segments of influenza A/H3N2 viruses indicated that the genome belonged to clade 3C.2a1b.2a.2b. and the occurrence of a reassortment event (Supplementary Fig. S2).

As persistent infections can lead to the emergence of viral variants characterized by altered transmissibility or immune evasiveness, intra-host viral diversity was assessed for both HCoV-OC43 and influenza H3N2 infections. The intra-host viral diversity

data suggested that the treatments impacted viral diversity of influenza virus, but not of HCoV-OC43 (Fig. 2, Supplementary Figs. S3 and S4).

In order to understand the treatments failures and evaluate options, high throughput whole genome sequencing was performed to obtain snapshots of the viral population. Prior to oseltamivir treatment, on D46, the virus presented wild-type (WT) genomic features. On day 54, two substitutions of interest in the neuraminidase coding segment were detected in the viral population: R292K at a frequency of 56% and E119V at a frequency of 36% (Fig. 1A). The short reads sequencing approach used did not allow to infer potential linkage between these changes, which may have been carried by distinct RNA genomes. The E119V substitution has been shown to confer reduced or highly reduced susceptibility to oseltamivir but no impact on zanamivir efficacy [20], and our *in vitro* assay on the viruses isolated at the end of the treatment confirmed the highly reduced phenotype in this recent H3N2 genotype context (2b) (Fig. 3). The R292K mutation has been associated with highly reduced susceptibility to oseltamivir. Its effect on zanamivir efficacy appears more variable among influenza viruses diversity, with reports for some influenza A subtypes, not including H3N2 viruses [21–23]. On Day 110, the R292K mutation was undetectable, but a deletion (del245–248) was identified at 100% frequency (Fig. 1B), which has been shown to confer high level of resistance to oseltamivir but no resistance to zanamivir [20]. This deletion was not detected as a minor variant population in prior time points.

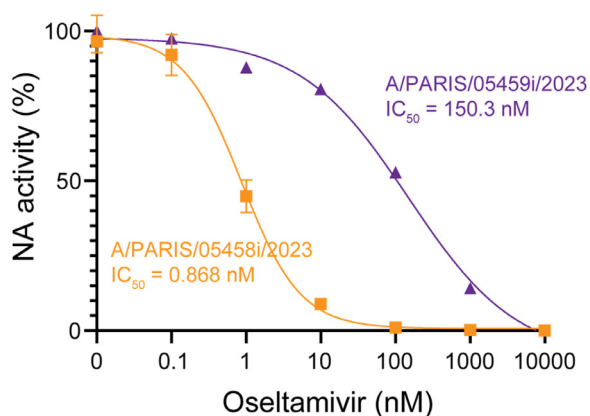


Fig. 3. Antiviral susceptibility. The 50% Inhibitory concentration (IC₅₀) of oseltamivir for viruses isolated prior to (A/PARIS/05458i/2023, WT) and at the end (A/PARIS/05459i/2023) of the oseltamivir treatment. Metatranscriptomic sequencing revealed that the A/PARIS/05459i/2023 isolate contains a viral population with 84% of the E119V substitution (up from 36% in the primary sample), and below 1% of R292K (down from 56% in the primary sample), suggesting a replication advantage of E119V *in vitro*, and compatible with previous reports indicating that the R292K negatively impact viral fitness *in vitro*.

3.4. Anti-influenza combination therapy

In the context of persisting pancytopenia not improving despite growth factors and romiplostim treatment, without suspicion of relapse or graft failure (98% donor chimerism), a CD34-HSCT boost or a second alloHSCT were discussed but considered unfeasible in view of the persisting viral infection. Moreover, the obstructive respiratory disorder on PFTs was suspected to be triggered by persistent respiratory viral infection.

In the aim of eradicating the influenza A infection, a new anti-viral course was started from D110, associating intra-venous zanamivir (600mg/day) and oral baloxavir-marboxil (40 mg once every 3 days, for a total of 4 doses). At the end of this treatment (D131), symptoms persisted with cough and pan-sinusitis, but the nasopharyngeal swab was negative for influenza A and surprisingly also for HCoV-OC43 (Fig. 1A, Supplementary Fig. 5) and remained negative for the four following weekly controls until D168, except weak influenza virus detection on D138. Oral azithromycin (250 mg thrice weekly) was started from D140 and followed by clinical improvement within 10 days. D154 and D204 PFTs were normal, arguing in favour of a viral-related obstructive pattern rather than a post-alloHSCT bronchiolitis obliterans. Haematological parameters slowly improved and no CD34-HSCT boost or second alloHSCT has been performed until now.

4. Discussion

Long-term shedding of influenza virus [3], SARS-CoV-2 [24] and other respiratory viruses [1] is a common occurrence in immunocompromised individuals due to impaired adaptive immune responses, leading to incomplete viral clearance. Such prolonged infections have been occasionally associated with rapid viral evolution and could represent a source of immune escape variants, as proposed for SARS-CoV-2 [25].

Our patient had undergone several courses of immunosuppressive treatments and experienced poor graft function after alloHSCT, resulting in a significantly compromised immune function, which proved insufficient in controlling influenza and HCoV-OC43 infections. Considering her underlying haematological condition and the potential need for a second alloHSCT, as well as the potential impact of prolonged viral shedding on pulmonary function, eradicating the influenza virus became imperative.

Consecutive treatments with oseltamivir and zanamivir were ineffective in controlling viral replication and respiratory symptoms. The decrease in influenza load during zanamivir treatment raises the question of whether extending the treatment beyond 10 days could have cured the infection. Considering the recommended duration of zanamivir treatment, the medical condition of the patient, and the risk of acquiring zanamivir resistance, as previously observed in similar conditions [2], it was decided to discontinue monotherapy and consider combined therapy to optimize the chances of a cure. Furthermore, during each treatment, resistant strains emerged. Oseltamivir led to the emergence of oseltamivir-resistant mutations in the neuraminidase, specifically R292K and E119V, although at frequencies of 56% and 36%, respectively in the captured snapshot, suggesting potential fitness issues. Sequencing during zanamivir treatment revealed that the E119V change had disappeared, and the proportion of R292K dropped to 6%. A deletion (del245-248, absent from previous sequencing) then emerged in the neuraminidase (Fig. 1B). Two reports showed this deletion being selected after either oseltamivir treatment [26] or successive oseltamivir and zanamivir treatments [27]. Surprisingly, while this deletion has been shown to confer a high level of resistance to oseltamivir, it does not affect zanamivir sensitivity significantly *in vitro* [26,27]. The persistence of selective pressure with zanamivir, combined with the introduction of baloxavir, may have contributed to the effectiveness in controlling influenza. A previous report described the success of a combination of zanamivir and baloxavir in a lung transplant recipient with oseltamivir-resistant H1N1 influenza infection [28]. Two other reports in HSCT recipients showed that baloxavir, as monotherapy, was effective in controlling influenza infection after oseltamivir or peramivir treatment failure for some patients, but not all [29]. A randomized controlled trial in patients hospitalized for severe influenza infections [4] showed that the combination of baloxavir and NAIs did not lead to superior clinical outcomes compared with NAIs alone. However, it did result in a faster reduction in viral load and a lower rate of emergence of antiviral drug resistance. The patient had a concomitant HCoV-OC43 infection that was controlled simultaneously with influenza. The clearance of HCoV-OC43 might have resulted from respiratory mucosal restoration due to influenza replication control and/or an inhibition of the main protease of HCoV-OC43. While the clinical antiviral efficacy of NAIs against coronaviruses appears limited [30], their inhibition of the host neuraminidase might contribute to the reduction of neutrophil overactivation [31]. Furthermore, in a mouse model, NAI treatment led to decrease in lung neutrophil infiltration, viral load and tissue damage following a beta-coronavirus challenge [31]. Despite *in silico* and *in vitro* data suggesting a potential activity of baloxavir against SARS-CoV-2 [32], there is no clinical evidence of efficacy against SARS-CoV-2 [33] and no data are available for other coronaviruses. Overall, these observations underscore the risk and complexity of selecting resistant viruses in deeply immunocompromised patients treated with neuraminidase inhibitors as monotherapy. They also highlight the need to continue evaluating combination treatments as the first line of treatment in those infected patients at high risk for acute and chronic complications.

Data availability

All data supporting the findings of this study are available in the manuscript.

Author contributions

Victor Euzen: Patient care, formal analysis, initial draft. Alienor Xhaard: Patient care, initial draft. Samar Berreira-Ibraim: Formal analysis. Laure Deville: Patient care, antiviral drugs supply and

dispensations. Aude Quentin: Patient care. Pedro Hendrique De Lmia Prata: Patient care. Viviane Gournay: Patient care. Matthieu Prot: Metatranscriptomic sequencing. Yannis Rahou: Isolation, IC50 and amplicon sequencing. Marion Barbet: Isolation and amplicon sequencing. Séverine Mercier-Delarue: Virus quantification. Régis Peffault De La Tour: Patient care. Etienne Simon-Loriere: Formal analysis, funding acquisition, supervision, manuscript writing. Jérôme Le Goff: Patient care, formal analysis, funding acquisition, supervision, manuscript writing.

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Declarations

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Competing interests: None.

Ethical approval: The patient gave her written consent for data collection.

Sequence information: HCoV-OC43 genome sequence deposited in GenBank (accession number OR371530), and the A/H3N2 genomes were deposited on GISAID (EPI_ISL_17512135, EPI_ISL_17512136, EPI_ISL_17512162 and EPI_ISL_17975828).

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at [doi:10.1016/j.ijantimicag.2024.107281](https://doi.org/10.1016/j.ijantimicag.2024.107281).

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