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Sequence analysis of respiratory syncytial virus cases reveals a novel subgroup -B strain circulating in north-central Italy after pandemic restrictions

Alessandra Pierangeli [a,*], Fabio Midulla [b], Antonio Piralla [c], Guglielmo Ferrari [c], Raffaella Nenna [b], Antonino Maria Guglielmo Pitrolo [c], Amelia Licari [d,e], Gian Luigi Marseglia [d,e], Dario Abruzzese [e], Laura Pellegrinelli [f], Cristina Galli [f], Sandro Binda [f], Danilo Cereda [g], Matteo Fracella [a], Giuseppe Oliveto [a], Roberta Campagna [a], Laura Petrarca [b], Elena Pariani [f], Guido Antonelli [a,h,1], Fausto Baldanti [c,d,1]

[a] Virology Laboratory, Department of Molecular Medicine, Sapienza University, V.le Porta Tiburtina, 28, 00185 Rome, Italy
[b] Department of Pediatrics and Infantile Neuropsychiatry, Sapienza University, V.le Regina Elena, 299, 00161, Rome, Italy
[c] Microbiology and Virology Department, Fondazione IRCCS Policlinico San Matteo, Viale C. Golgi, 19, 27100 Pavia, Italy
[d] Department of Clinical, Surgical, Diagnostic and Pediatric Sciences, University of Pavia, via S. da Nuova, 65, 27100 Pavia, Italy
[e] Pediatric Clinic, Fondazione IRCCS Policlinico San Matteo, Viale C. Golgi, 19, 27100 Pavia, Italy
[f] Department of Biomedical Sciences for Health, University of Milan, via C. Pascal, 36, 20133 Milan, Italy
[g] DG Welfare, Regione Lombardia, Piazza Città di Lombardia, 1, 20124, Milan, Italy
[h] University Hospital Policlinico Umberto I, Sapienza University, V.le del Policlinico 155, 00161 Rome, Italy

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ABSTRACT

Background: Following the pandemic restrictions, the epidemiology of respiratory syncytial virus (RSV) has changed, leading to intense hospitalization peaks.
Objectives: This study, conducted at multiple sites in Italy, aimed to describe the temporal dynamics of two post-COVID-19 RSV epidemics. Additionally, the circulating RSV-A and -B lineages were characterized and compared to those found in 2018 and 2019.
Study design: Respiratory specimens and data were collected from RSV-positive patients, both inpatients, and outpatients, of all ages at three sites in north-central Italy. To analyze these samples, roughly one-sixth were sequenced in the attachment glycoprotein G gene and subjected to phylogenetic and mutational analyses, including pre-pandemic sequences from north-central Italy.
Results: The first post-pandemic surge of RSV cases was quite intense, occurring from October 2021 to early January 2022. The subsequent RSV epidemic (from November 2022 to early March 2023) also had a high impact, characterized by a rise in elderly patient cases. Post-pandemic cases of RSV-A were caused by various strains present in Italy prior to COVID-19. In contrast, a distinct RSV-B lineage, which was concurrently spreading in other countries, was identified as the main cause of the surge in 2022–2023 but remained undetected in Italy before the pandemic.
Conclusions: This study describes the temporal dynamics of post-pandemic RSV subgroups and uncovers a lineage of RSV-B with high genetic divergence that may have increased the impact of decreased population immunity.

Abbreviations: RSV, respiratory syncytial virus; COVID-19, corona virus disease-2019; SARS-CoV-2, severe acute respiratory syndrome-coronavirus-2; HVR, highly variable region; CCD, central conserved domain; MI, Milan; PV, Pavia; RM, Rome; ILI, influenza-like illness; ARI, acute respiratory infection; PCR, polymerase chain reaction; HRV, human rhinovirus; GISAID, Global Initiative on Sharing All Influenza Data.

* Corresponding author at: Virology Laboratory, Department of Molecular Medicine, V.le Porta Tiburtina, 28; 00185 Rome, Italy.
E-mail address: alessandra.pierangeli@uniroma1.it (A. Pierangeli).

[1] The role of senior author is shared by these authors.

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1. Introduction

During the first phase of the COVID-19 pandemic, respiratory viruses - including respiratory syncytial virus (RSV) - circulated at low rates, as a result of the non-pharmaceutical interventions (NPIs) used to control SARS-CoV-2 [1]. Reduced infections resulted in waning population immunity ("immunity debt") [2,3]; after NPIs were reduced, RSV caused a peak in hospitalizations in the summer-fall of 2021 [4–6]. In 2021, influenza virus circulated at low levels with a small peak in early spring 2022 [7]. In the fall of 2022, RSV circulation spiked again, along with an intense circulation of influenza virus; together with SARS-CoV-2, the three viruses caused a "triple demic" with a severe impact on health services [8].

In the pre-pandemic period, seasonal RSV epidemiology was characterized by a not well-defined alternation of subgroups A and B and their genotypes [9,10] and differences in virulence between genotypes/strains have been reported [11,12], although these issues remain controversial [13]. RSV classification is currently being reconsidered according to either full-genome sequences [14,15] or those of the G gene [16,17], traditionally used for classification [18]. The G glycoprotein consists of a cytoplasmic tail, a transmembrane domain, a first highly variable region (HVR1), a non-glycosylated central conserved domain (CCD) containing the so-called cysteine noose, i.e. a tract with four conserved cysteines including those of the CX3C chemokine motif, and the heparin binding site, followed by the second HVR (HVR2) up to the stop codon [19,20]. In the recent RSV evolutionary history, novel genotypes have emerged that are characterized by major changes in G, namely an insertion of 72 (RSV-A) [21] or 60 (RSV-B) [22] nucleotides in the HVR2 duplicating 23 or 19 amino acids (aa), respectively. These genotypes, initially named RSV-A ON1 and RSV-B BA, soon [23] or gradually [24] replaced the previously circulating ones and continued to differentiate [25,26]. RSV genetic evolution may have been affected by the COVID-19 pandemic [1]; the decline in infections may have caused a genetic bottleneck, resulting in the extinction of pre-pandemic lineages and the emergence of a limited number of RSV strains with local variations, as seen in Australia [27].

The aim of this study was to characterize RSV epidemiology in north-central Italy, comparing RSV-positive cases between 2021–2022 and 2022–2023, and to investigate RSV variants in the post-pandemic seasons.

2. Materials and methods

2.1. Patients and samples

The study included RSV-positive respiratory specimens tested in three virology laboratories, located in Milan (MI) and Pavia (PV), northern Italy, and in Rome (RM), central Italy, during the periods September–April 2021–2022 and September-April 2022–2023. MI University, which participates in the National Influenza Sentinel Surveillance Network (InfluNet), tested nasopharyngeal swabs from outpatients of all ages with influenza-like illness (ILI). In PV, respiratory virus testing was performed on patients with acute respiratory infection (ARI) at the Fondazione IRCCS Policlinico San Matteo, at the request of the attending physician. In RM, RSV testing was performed on patients with ARI admitted to the Pediatric Emergency Department of the Sapienza University Hospital and other departments of the same hospital. The institutional review board and the Ethics Committee of Sapienza University Hospital approved the study (Prot. 107/12). Patient demographics were retrospectively extracted from medical records and anonymized.

2.2. RSV detection and sequence analysis

In the three centers, different lab-developed, qualitative PCR or real-time PCR methods were used to detect RSV and other respiratory viruses, for RSV subgrouping, and for sequencing as reported in Supplementary Methods including Table S1.

RSV-A and -B positive samples were randomly selected for G gene Sanger sequencing and results were aligned to reference sequences using Bioedit.7.1.3; sequencing errors were removed and redundant sequences from the same site and from the same epidemic season were grouped. In addition, sequences from strains circulating in MI, PV, and RM during the 2018–2019 season [28] and 2019–2020 RM sequences obtained for this study were included in the analysis.

The phylogenetic tree was constructed on IQ-TREE [29] using the Maximum Likelihood method based on the General Time Reversible model and discrete Gamma distribution with 5 categories, with bootstrap values of 1000. Goya et al's classification and reference genotypes from their paper [17] were used.

Translated study sequences and global strains were aligned to the RSV-A GA2.3.5 (MH447952.1_TH/C3208/2012) and to the RSV-B GB5.0.5a (KY249660.1_England583/2013) strains.

The GISAID [https://gisaid.org/] and NextStrain [https://nextstrain.org/rsv] websites were accessed in September 2023, to compare patterns of aa substitutions.

The bioinformatic tools NetNGlyc and NetOGlyc [http://www.services.healthtech.dtu.dk] were used to predict N- and O-linked glycosylation sites.

2.3. Statistical analysis

Chi-squared was used to test the statistical difference for categorical variables; the Student *t*-test or Mann-Whitney test was used to compare quantitative variables. Statistical analyses were performed using SPSSv27 (SPSS Inc. Chicago, IL, USA), and significance was set at $p < 0.05$.

3. Results

3.1. RSV weekly distribution and subgroup prevalence

A total of 1304/10,689 (12.2 %) cases were positive for RSV from September-April 2021–2022 and September-April 2022–2023. Of 3882 respiratory specimens tested in 2021–2022, 588 (15.1 %) were RSV positive. The absolute number of cases testing positive for RSV increased to 716 in 2022–2023, but, as the number of specimens tested nearly doubled, the RSV positivity rate decreased significantly (Table 1). This trend was not observed in Rome, where the RSV positivity rate remained constant, despite a substantial increase in the number of specimens tested in 2022–2023 (Table S2). The coinfection rate calculated in approximately two-thirds of the samples was similar in the two seasons. The most common coinfecting virus was rhinovirus (HRV) in both seasons, but RSV/HRV cases decreased from 61 % in 2021–2022 to 38.5 % in 2022–2023 (Table 1 and S2), when more coinfections with other respiratory viruses (influenza, parainfluenza, adenovirus, metapneumovirus and seasonal coronaviruses) were detected (data not shown).

The age of RSV-positive subjects was higher, and adults accounted for a larger proportion of the RSV-positive cases in 2022–2023 than in 2021–2022 (Table 1); despite the different enrollment criteria and a significantly different age of RSV-positive cases (Table S2), this trend was the same among the three sites.

MI University tested ILI outpatients for respiratory viruses, 1181 specimens from September 2021 to April 2022 and 2378 in the following season (Table S2); RSV-positive cases were equally numerous in the two study periods, but the positivity rate decreased significantly in the 2022–2023 season as the number of specimens tested doubled (Fig. 1 and Table S2). Among the 5152 cases tested in the PV hospital, the RSV-positive cases were comparable in the two seasons; the RSV positivity rate decreased significantly in 2022–2023, but the RSV hospitalization rate did not change (Fig. 1 and Table S2). In contrast, in Rome, the RSV

Table 1
Demographic characteristics and subgroup distribution of RSV-positive cases identified from September 2021 to April 2022 and from September 2022 to April 2023 in north-central Italy.

RSV-positive samples		2021–2022	2022–2023	p value
RSV-positive samples		588/3882 (15.1 %)	716/6807 (10.5 %)	<0.0001
RSV in coinfection[a]		136/380 (35.7 %)	148/434 (34 %)	0.663
RSV/Rhinovirus coinfections		83/136 (61.0 %)	57/148 (38.5 %)	0.00015
Female/Male[b]		236/279	327/332	0.196
Mean age in years[b] (SD)		9.2 (20.1)	17.8 (29.1)	<0.0001
Age group[b]	< 1 year	222	249	<0.0001
	1–5 years	185	191	
	6–16 years	24	51	
	17–60 years	50	62	
	>60 years	34	106	
Adult patients[b] (≥18 years)		84/515 (16.3 %)	168/659 (25.5 %)	0.0001
RSV-A-positive samples (%)[c]		253/473 (53.5 %)	108/508 (21.3 %)	<0.0001
RSV-B-positive samples (%)[c]		220/473 (46.5 %)	400/508 (78.7 %)	

[a] Data on coinfections are available from the number of samples reported as the denominator.
[b] Data on sex assigned at birth and on age are available for 515/588 in 2021–2022 and for 659/716 RSV-positive cases in 2022–2023.
[c] The denominator is the number of samples in which the subgroup was determined.

positivity rate among patients hospitalized for respiratory illness remained constant despite a significant increase in the number of specimens tested in 2022–2023 (Fig. 1 and Table S2).

The weekly distribution of RSV-positive cases as a percentage of tested specimens is shown in Fig. 1 for each center. The general epidemic trends were similar for the three regions: in 2021–2022, RSV circulation started in October 2021, peaked in November (earlier in Milan, later in Rome), and ended in January 2022 while, in 2022–2023 it started in November, had sustained circulation in December and January, and ended in early March.

The subgroup could be identified in most of the RSV positive samples (981/1299: 75.5 %). Overall, in 2021–2022, RSV-A caused more infections (53.5 %) than RSV-B, but in MI, RSV-B was more prevalent (58.4 %) than RSV-A (Table S1). In contrast, RSV-B largely dominated (78.7 %) in 2022–2023 (Table 1) and in MI, the RSV-B positivity rate increased to 85.9 % (Table S1).

3.2. Sequence analysis

From RSV-positive cases of any age, 217/1304 (16.6 %) were randomly sequenced; data from 133/217 cases are shown in Table S3. For phylogenetic analyses, the RSV-A dataset contained 59 post-pandemic sequences (from 108 cases) and 46 pre-COVID-19 sequences; the RSV-B dataset contained 81 post-pandemic sequences (from 109 cases) and 35 pre-COVID-19 sequences.

All RSV-A sequences carried the 72-nucleotide insertion at position 850–921 (aa 284–307) of the G gene; however, they were evolutionarily distant from the prototype ON1 strain [21]. Study sequences clustered with lineages GA2.3.5, GA2.3.6a, GA2.3.6b [17]; interestingly, 34 pre- and post-pandemic sequences grouped into a divergent clade derived from GA2.3.5 (evidenced in Fig. 2).

Most nucleotide substitutions, mainly non-synonymous, were found in the HVRs, especially in the HVR2. The CCD tract was conserved, with the exception of a two-nucleotide mutation (AA to GG) altering the conserved asparagine (N) at aa position 178 (Fig. 3A). The signature aa substitutions in sequences clustering with lineage GA2.3.6b are: A57V (found in only one pre-pandemic sequence), P206Q, L248I, and V303A (all found in several 2018–2019 and 2019–2020 strains, Fig. 3A). In GISAID, this pattern of aa changes was found in more than 500 sequences, including some from 2019-2020. The more divergent clade, including 7/46 pre-pandemic and 13/59 post-pandemic study sequences, is characterized by T113I, V131D, N178G, H258Q, H266L, a pattern found in more than 1250 sequences in GISAID. In addition, a cluster of nine 2022–2023 RM cases represented by two sequences (eight were identical), have Y273H, and S277P, and Y280H. Another notable monophyletic node of seven 2022–2023 PV sequences (five were identical) was characterized by an aa substitution, A65T, located at the end of the transmembrane domain, which was found in only 24 pre-pandemic GISAID sequences. The occurrence of these monophyletic nodes with high bootstrap support suggest further local divergence of RSV-A strains.

Almost all RSV-A study strains were predicted to have three N-glycosylation sites as the ON1 ancestor; the N103T substitution, found in two identical 2021 RM sequences and in a 2022 US strain [30], resulted in the loss of one N-glycosylation site whereas S299N in a pre-pandemic and in a post-pandemic strain potentially introduced a fourth N-glycosylation site. There was no considerable difference in the predicted O-glycosylation sites among RSV-A strains across various epidemic seasons and clades (data not shown).

All RSV-B study sequences carried the 60-nucleotide insertion at position 775–834 of the G gene (aa 259–278); nine sequences obtained in this study from pre-pandemic cases were genetically close to the GB5.0.5a prototype and other pre-pandemic and post-pandemic sequences clustered with the GISAID reference sequence (hRSV/B/Australia/VIC-RCH056/2019). Interestingly, most of the post-pandemic and none of the pre-COVID-19 sequences formed a large divergent clade (evidenced in Fig. 2B) clustering with 2021 and 2022 strains from the US [30–32], and Austria [33]; the latter study identified these divergent RSV-B strains as a new lineage, GB5.0.6a [33].

In comparison with the GB5.0.5a prototype, RSV-B study sequences had several aa changes in HVR1, no substitution in the CCD, and higher variability in HVR2, which was even more remarkable in the divergent clade (evidenced in Fig. 3B). hRSV/B/Australia/VIC-RCH056/2019 differed from the GB5.0.5a prototype for four signature changes, A131T, T137I, T290I and T312I, found in pre-pandemic ($N = 27$) and post-pandemic ($N = 18$) study sequences, together with several other substitutions (Fig. 3B). The divergent RSV-B clade containing 16 2021–2022 and 49 2022–2023 study sequences, is characterized by a pattern of six/seven changes in the HVR2, including P216S, P223L, I252T, K258N, I272T, S277P and Y287H. In addition, S100G is present in most strains, apart from the 2022–2023 PV sequences in which 100G, found in 2021–2022, has reverted to S100; moreover, K209N arose in a monophyletic node formed by 9 identical RM sequences.

The predicted number of N-glycosylation sites in RSV-B differed more than in RSV-A: in the divergent clade, sequences carrying the K258N substitution acquired a fourth predicted N-glycosylation site, whereas the other strains had three N-glycosylation sites like the GB5.0.5a prototype. In addition, K209N led to a further potential N-glycosylation site in the nine identical 2022–2023 RM sequences. Predicted O-glycosylation sites did not differ among RSV-B strains (data not shown).

4. Discussion

This study compared the first two RSV epidemics after COVID-19 and characterized the RSV strains circulating in north-central Italy. In 2021–2022, a high number of RSV-positive cases in ILI and in hospitalized patients was recorded over a relatively short RSV season that peaked in November. After the removal of all remaining restrictions in the summer of 2022, the RSV epidemic was again quite intense, but with a temporal distribution similar to the typical RSV seasonality, starting in November, peaking in December/January, and ending in March. The number of RSV-positive cases increased, despite the concurrent intense

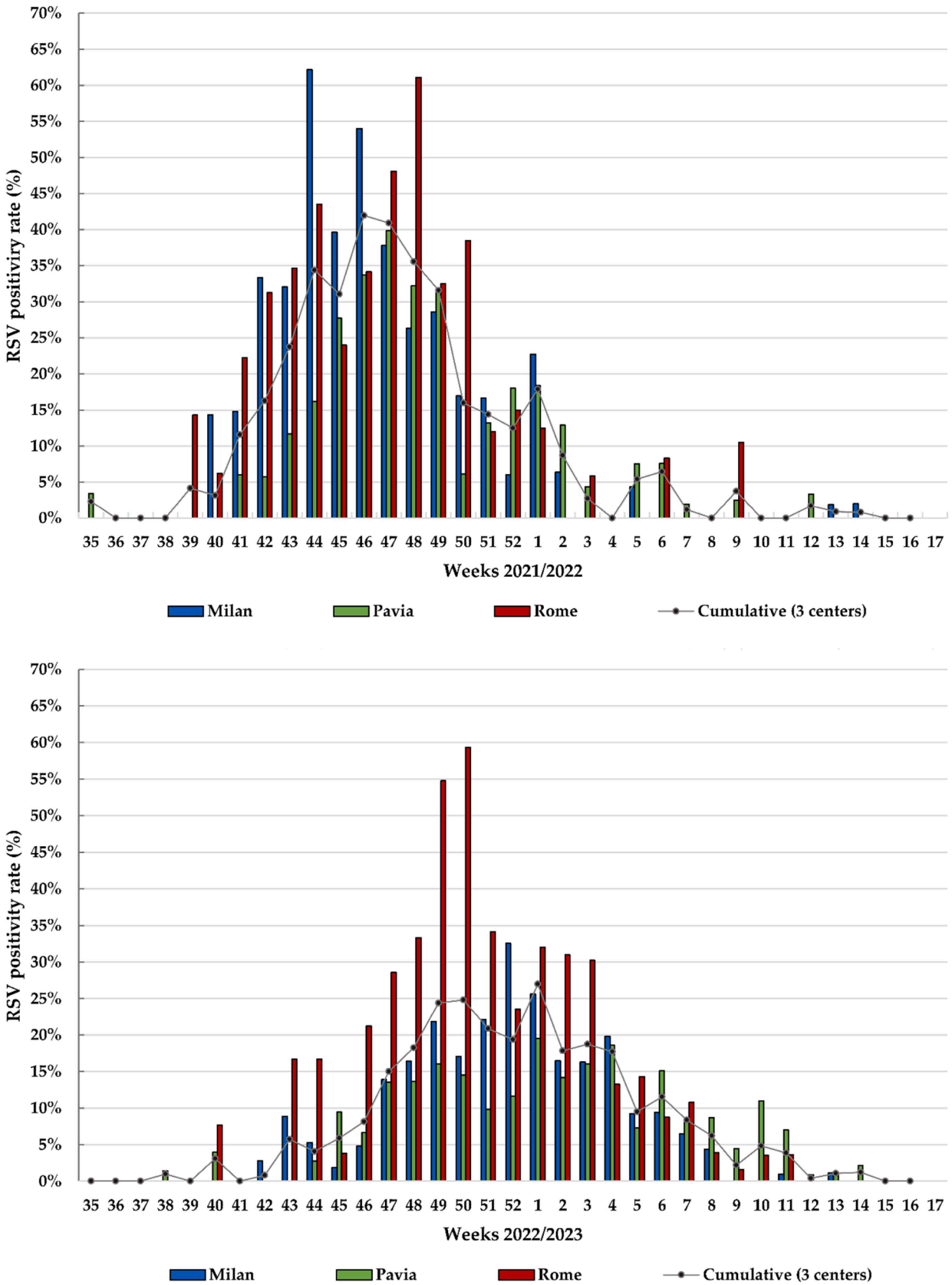

Fig. 1. Weekly distribution of the total number of respiratory specimens tested and the respiratory syncytial virus (RSV)-positive cases in each center. The upper part shows the weekly distribution of total respiratory specimens tested and RSV-positive cases for each center during September 2021-April 2022, and the lower part during September 2022-April 2023. The calendar weeks are plotted on the X-axis; the number of RSV-positive cases is plotted on the left Y-axis and the total number of respiratory samples tested is plotted on the right Y-axis.

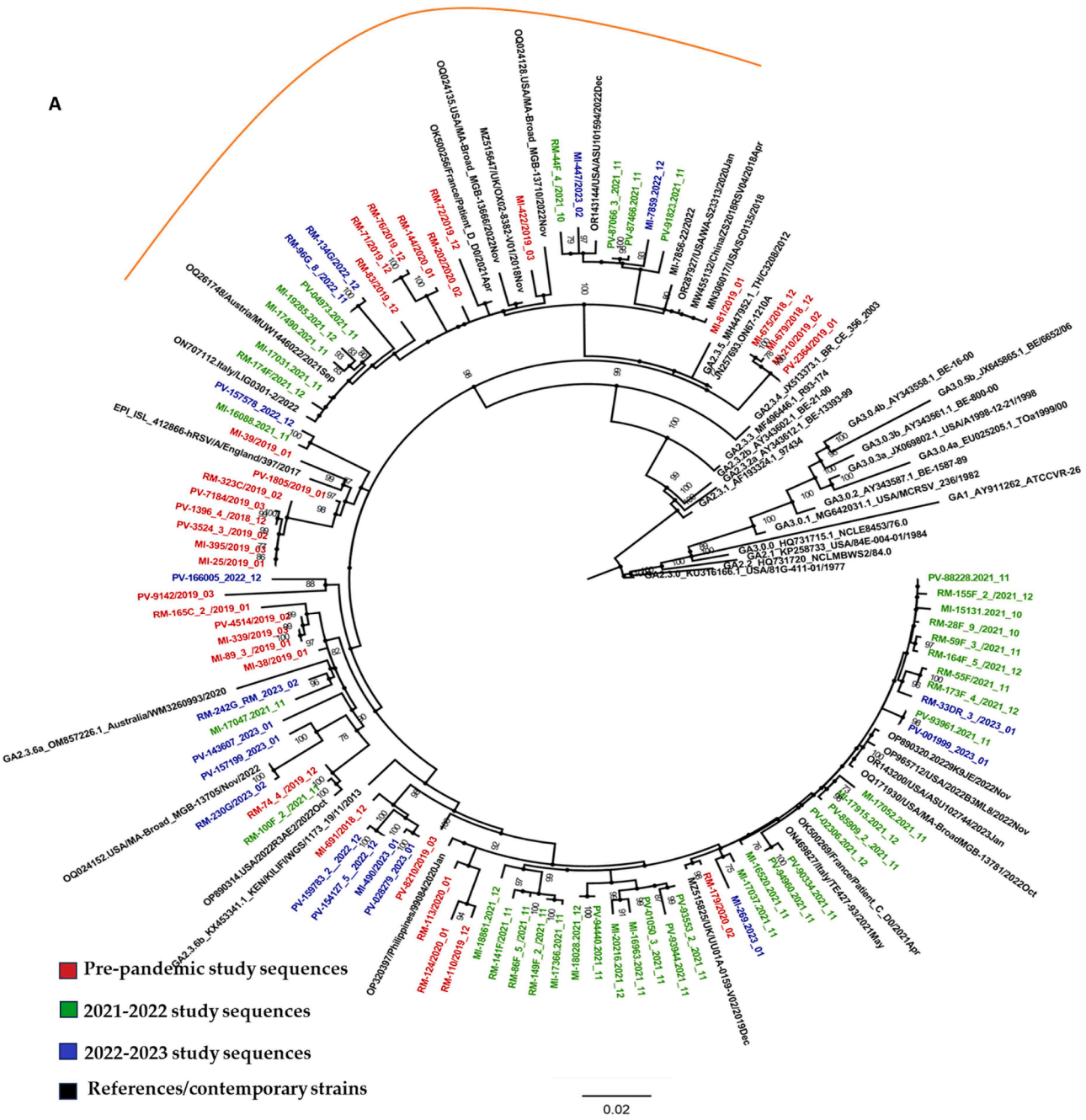

Fig. 2. Phylogenetic analysis of the G gene of RSV-A (A) and RSV-B (B) strains. Labels at branch nodes indicate bootstrap support >70 %. Scale bar indicates nucleotide substitutions per site. Study sequences are identified by the site (MI for Milan, PV for Pavia and RM for Rome), the specimen number followed by the year (4 digits) and month (2 digits). If more than one identical sequence was found, the total number is given in parentheses after the strain ID. The phylogenetic tree is drawn to scale, and a scale bar below the tree shows the number of substitutions per site. The divergent clades found for RSV-A and RSV-B are indicated by curved lines.

influenza virus circulation, confirmed by the marked increase in respiratory specimens tested. The higher number of respiratory infections in 2022–2023 in Italy is due to the return to a more sustained circulation of multiple respiratory viruses, also witnessed by a significant increase in viral co-infections other than RSV/HRV cases. Notably, in 2022–2023, RSV circulated comparatively more in the elderly who were more protected by residual NPIs in 2021–2022. However, the age breakdown of all subjects tested is not currently available to calculate the RSV positivity rate in each age group in the two seasons.

In several countries, the first post-pandemic surge of RSV cases was recorded in summer [5,34–36] or in autumn 2021 [33,37,38]; these RSV epidemics were less intense than expected and clinical severity was lower or similar to that in pre-pandemic seasons [5,34–40]. While the increase in RSV cases in 2021 was largely predicted [1,41], the intensity of the RSV epidemic in 2022–2023 was somewhat surprising in those countries where RSV was abundantly circulating in 2021 and the "immunity debt" was at least partially paid. Furthermore, the simultaneous circulation of respiratory viruses, including SARS-CoV-2, influenza, and RSV, was unexpected given the demonstrated interference between viruses [42,43]. Indeed, at the population level, viral competition for the

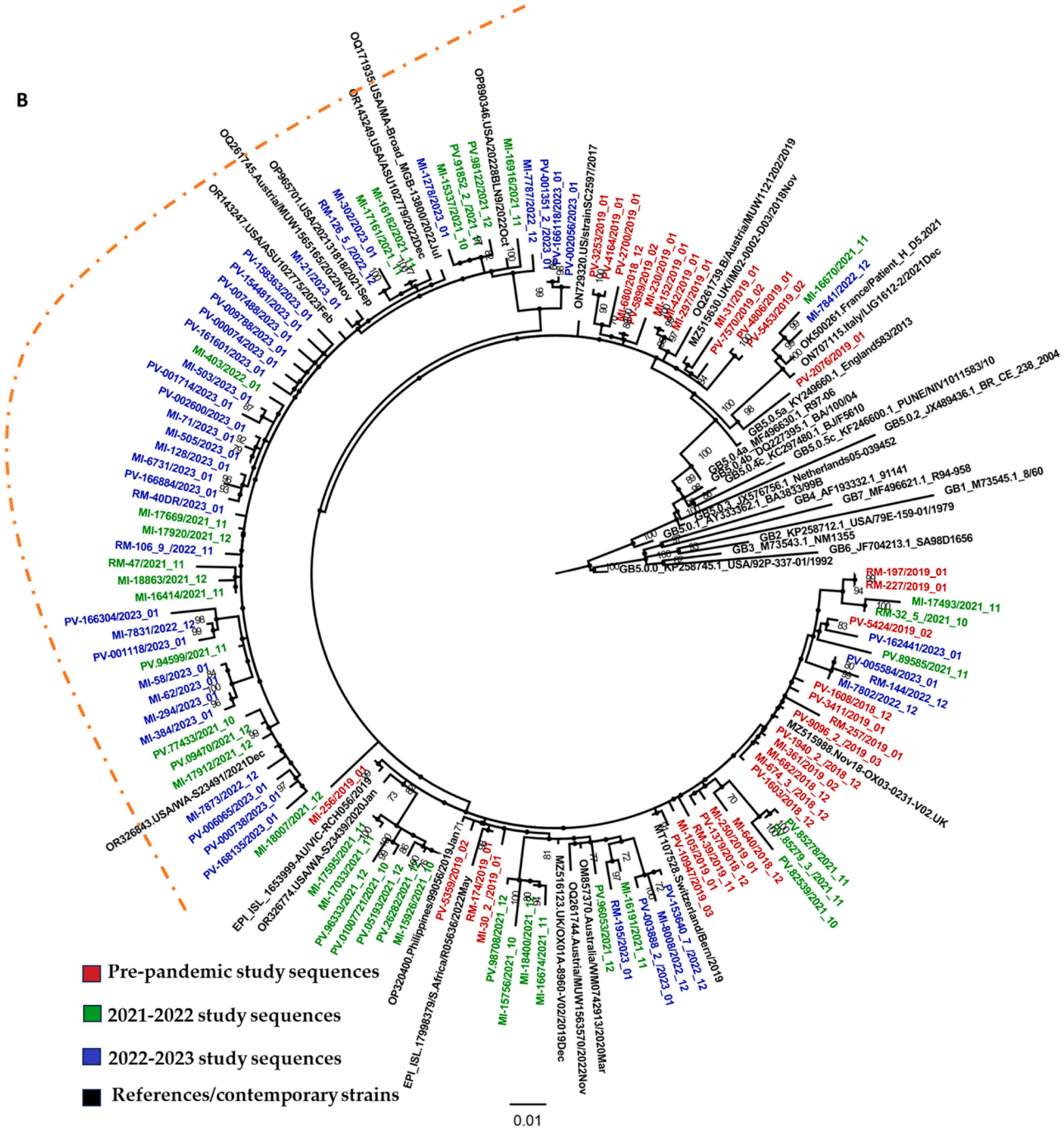

Fig. 2. (*continued*).

same host has shaped the epidemiology of seasonal and pandemic respiratory viruses [44,45]. Despite viral competition and the reduced RSV immunity debt [36], the 2022–2023 RSV epidemic was intense in several countries, where RSV hospitalizations were higher than in 2021–2022 [30–33,36,46]. To explain this unexpected RSV epidemiology, some authors have hypothesized that prior SARS-CoV-2 infection may confer an increased risk for RSV infection [47,48] or that divergent RSV strains may have emerged [48].

Here it was found that RSV-A prevailed over RSV-B during 2021–2022, whereas RSV-B had a greater occurrence in 2022–2023, which aligns with what has been documented in other European nations [33,37]. In the three sites, post-COVID-19 RSV-A and -B epidemics were not caused by a single predominant lineage surviving to restrictions but by strains that were genetically drifted with respect to those circulating before 2020, with substantial differences between subgroups. The post-pandemic RSV-A sequences belonged to different contemporary lineages, each of which included strains that were found also in the pre-pandemic period. The more divergent study clade was characterized by the N178G substitution which is part of the cysteine noose (position 176–186) in the CCD. Broadly neutralizing monoclonal antibodies [49,50] bind to epitopes that always include two conserved asparagines, N178 and N179; therefore, the N178G change may alter the affinity for preformed antibodies, and contribute to immune escape. Together with N178G, the four substitutions (T113I, V131D, H258Q, H266L) in the HVRs of the more divergent clade were identified in China in 2017–2018 [51,52]. The recurring substitutions Y273H, S277P, Y280H and several others, were found in global strains [27,31–34,53,54] as examples of multiple introductions of RSV-A strains in Italy.

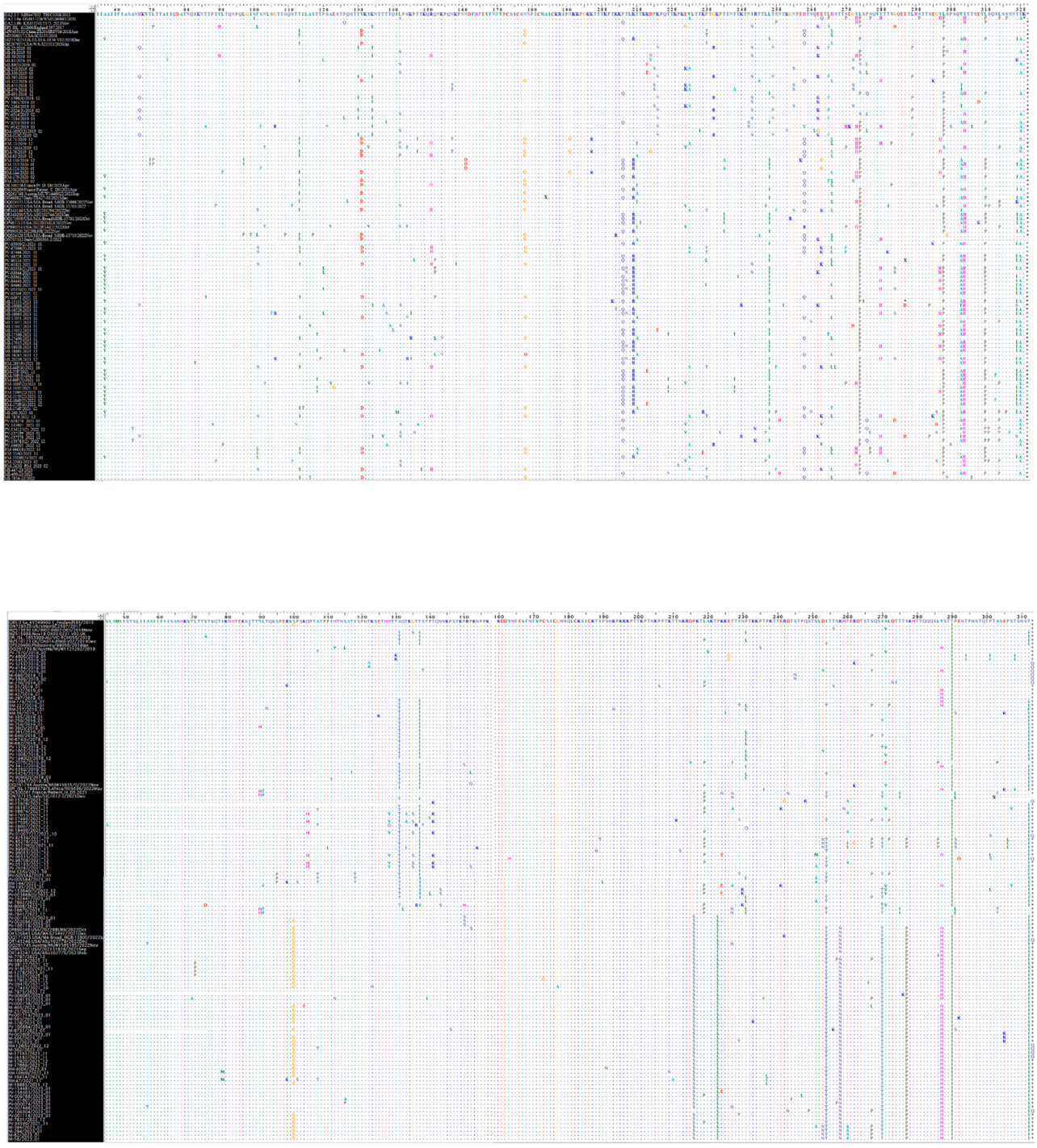

Fig. 3. Alignments of the G protein sequences of RSV study strains. Alignments of unique study sequences from Milan (MI), Pavia (PV) and Rome (RM), together with contemporary strains from different countries, are illustrated. Substitutions are shown relative to the RSV-A lineage GA2.3.5 (MH447952.1_TH/C3208/2012) and to the RSV-B lineage GB5.0.5a (KY249660.1_England583/2013) reference strains [17]. Dots indicate aa identical to the reference strain; the amino acids are colored according to BioEdit.7.1.3 codes for ease in visual identification of aa polymorphisms. In the alignments, pre-pandemic global strains are shown below the reference strain [17], followed by pre-pandemic study sequences, then post-pandemic global strains, and post-pandemic study sequences. Those belonging to the RSV-B divergent clade are grouped below.

RSV-B study sequences also belonged to different lineages; unlike RSV-A, no mutation in the CCD was found, but strains in the divergent clade had a mutational pattern undetected in pre-pandemic study sequences. While the CCD is structurally rigid, the two HVRs are highly glycosylated "mucin-like" domains rich in proline, serine, and threonine. It has been proposed [55] that as long as sufficient O-linked glycosylation is preserved, a relaxed selection acts on the HVRs, allowing many aa changes that drive the evolution of RSV-A and -B strains over time [26, 56]. Indeed, similar RSV-B divergent strains, characterized by the pattern S100G, P216S, P223L, were identified in the USA [32], in the UK [https://gisaid.org/] and in Austria [33], where they became predominant in 2022–2023; their origin was dated to 2019 [33]. In addition to the GB5.0.6a lineage signature pattern, the divergent sequences in this study contained K258N, which would confer an additional

N-glycosylation site, potentially interfering with antibody binding and/or altering glycoprotein conformation [57]. This clade, due to its genetic divergence and novelty in Italy, could have played a role in the upsurge of RSV-B cases. Furthermore, the RM group recently reported that RSV-B caused more severe bronchiolitis in 2022–2023 than in previous seasons [58]. In fact, genomic mutations can confer improved replicative fitness and altered viral pathogenicity, as in the SARS-CoV-2 "variants of concern". Indeed, a study limitation is the lack of whole genome sequencing which would have provided more data on the genetic diversity of post-pandemic RSV strains. However, RSV strains associated with severe epidemics have been identified in previous studies [11–13] by divergence in the G sequence.

In conclusion, this analysis showed that multiple RSV lineages were circulating in Italy after the pandemic period, with a notable difference between the subgroups. Post-pandemic RSV-A strains were found in Italy years before COVID-19, whereas the predominant RSV-B lineage had a very recent evolutionary origin and this novelty, together with RSV-B-specific immunity debt, may have fueled the intensity of the 2022–2023 epidemic. Monitoring the changing epidemiology and local evolution of RSV is essential to prevent diffuse and severe outbreaks and viral resistance to prophylactic measures.

CRediT authorship contribution statement

Alessandra Pierangeli: Conceptualization, Formal analysis, Writing – original draft. **Fabio Midulla:** Methodology, Supervision. **Antonio Piralla:** Formal analysis, Writing – review & editing. **Guglielmo Ferrari:** Investigation. **Raffaella Nenna:** Formal analysis, Resources. **Antonino Maria Guglielmo Pitrolo:** Investigation. **Amelia Licari:** Formal analysis, Resources. **Gian Luigi Marseglia:** Methodology, Supervision. **Dario Abruzzese:** Resources. **Laura Pellegrinelli:** Formal analysis. **Cristina Galli:** Investigation. **Sandro Binda:** Data curation. **Danilo Cereda:** Formal analysis. **Matteo Fracella:** Investigation. **Giuseppe Oliveto:** Investigation. **Roberta Campagna:** Investigation. **Laura Petrarca:** Resources. **Elena Pariani:** Formal analysis, Writing – review & editing. **Guido Antonelli:** Funding acquisition, Writing – review & editing, Methodology. **Fausto Baldanti:** Funding acquisition, Methodology.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jcv.2024.105681.

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