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Original article

# SARS-CoV-2, influenza, HRSV and other respiratory pathogens during the post-COVID-19 era: Epidemic circulation in Italy in the 2023/2024 season

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ABSTRACT

*Objectives:* This study aimed at describing the epidemiological aspects of respiratory pathogens involved in cases of severe acute respiratory infections (SARI) in Siena (Tuscany, Italy) during the 2023/2024 season.
*Methods:* Oropharyngeal swabs were collected from enrolled patients in the University Hospital Trust of Siena. Swabs were tested by qRT-PCR for the detection of SARS-CoV-2 and other 21 respiratory pathogens, including influenza and human respiratory syncytial virus (HRSV). Swabs positive for SARS-CoV-2 and/or influenza were further analyzed by Next-Generation sequencing (NGS).
*Results:* From January to June 2024, 138 patients diagnosed with SARI were enrolled, with an average age of 76.7 years and a frequent presence of comorbidities. Among the patients, 40 % tested positive for SARS-CoV-2, followed by 20 % positivity for influenza and 13 % positivity for HRSV. For SARS-CoV-2, variants belonging to the JN.1, EG.5 and BA.5 lineages were found. The influenza A(H1N1)pdm09 strains were found to belong to the 6B.1a.5a.2a clade, while for influenza A(H3N2), the strains belonged to two different clades (3 C.2a1b.2a.1a and 3 C.2a1b. 2a.2a.3a.1).
*Conclusions:* This study contributes to the understanding of the spread of respiratory viruses and the genetic characteristics of SARS-CoV-2 and influenza in patients with SARI, underlining the importance of continuous surveillance.


## Introduction

In Italy, as in other temperate regions of the Northern Hemisphere, winter waves of severe acute respiratory infections (SARI) caused by various pathogens are observed annually, including severe acute respiratory syndrome Coronavirus 2 (SARS-CoV-2), influenza, human respiratory syncytial virus (HRSV) and other respiratory viruses and bacteria.

SARS-COV-2 was first identified in December 2019 in Wuhan, China [1]. Due to its rapid global spread, on 11 March 2020 it was declared a pandemic by the World Health Organization (WHO) [2], with an end date of 3 May 2023. As of today, in Italy, there has been almost 27 millions of confirmed cases and more than 197,000 deaths [3]. The global vaccination campaigns, which started in 2021, aimed primarily at immunizing at-risk populations and then extending to the rest of the population. Variants of the virus, which have emerged because of mutations, have further complicated pandemic management, requiring continuous monitoring activities and adaptation of prevention strategies. According to data published by the Ministry of Health, as of September 2023, in Italy 91.7 % of the population over 12 had been vaccinated with at least one dose, while 90.25 % had completed the vaccination cycle. Booster doses were administered to

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84.9 %, 16.9 % and 8.6 % of the potential vaccine targeted population, respectively [4]. In the autumn of 2022, the administration of the bivalent Comirnaty Original/Omicron BA.4–5 vaccine was authorized, mainly as a booster dose for individuals at high risk of developing severe forms of COVID-19, including the over 60 years and people with specific risk factors [5]. In the following season, an updated vaccine for the Omicron XBB.1.5 variant was introduced to extend protection against new circulating variants.

Influenza is a respiratory disease caused by influenza viruses type A (IAV) and type B (IBV). The WHO estimates that seasonal influenza is associated with one billion cases each year, including 3–5 million cases of severe illness and 290,000 to 650,000 deaths annually [6]. In industrializes countries, most influenza-associated deaths occur in people over 65 years of age and/or people with underlying chronic diseases [7]. The Italian Ministry of Health recommends anti-influenza vaccination every year, especially for children, elderly, people with chronic diseases as subject with severe forms, although the entire population can develop serious complications, including pneumonia, myocarditis and encephalitis which can lead to death [8]. However, despite the recommendations, influenza vaccination coverage remains low for both the general population and older adults in Italy. According to data on vaccination coverage in Italy only 18.9 % of the general population and 53.3 % of the elderly have been vaccinated for the 2023/2024 flu season [9].

HRSV is among the top four viral causes of Influenza-like illness (ILI) (third before the advent of SARS-CoV-2) after enterovirus and influenza and is the second most common cause of hospitalization [10–12]. Commonly, it is the leading cause of lower respiratory tract infections in children under one year of age [13], especially bronchiolitis and pneumonia and one of the most important in adults over 65 years of age and people with at-risk conditions [14,15]. During the 2019/2020 season, 26.6 % of hospitalized Italian adults with respiratory symptoms were diagnosed with HRSV [16]. Since the winter of 2022/2023 the HRSV has had an epidemiological change which has prompted experts to expand monitoring both towards the target population and to extend control not only to the winter months but also in autumn and spring.

In Italy, influenza surveillance takes place through a surveillance system coordinated by the Istituto Superiore di Sanità, and the contribution of the Ministry of Health. General practitioners and pediatricians of free choice participate on a voluntary basis in this surveillance network for ILI cases. Since the advent of the SARS-CoV-2 pandemic, this influenza surveillance system has evolved and changed its name, from InfluNet to RespiVirNet, precisely to deal with the circulation of other respiratory viruses that were previously monitored mainly in certain age groups, such as HRSV. The RespiVirNet operating protocol season 2023/2024 required that the laboratories operating in the network test systematically swabs for SARS-CoV-2 and other respiratory viruses, including HRSV [17]. Since the A/H1N1 influenza pandemic of 2009/2010, monitoring of severe forms of influenza in hospitalized subjects has also been active.

In response to COVID-19 pandemic, many countries, including Italy, implemented non-pharmaceutical measures, such as wearing masks, social distancing, and school and airport closures. These actions altered the seasonal transmission of airborne viruses, including influenza and HRSV [18,19]. In Italy, the 2020/2021 influenza season saw only the circulation of SARS-CoV-2, while the surveillance system did not record the circulation of influenza viruses [20,21]. The 2021/2022 season showed increased influenza incidence, with a bimodal ILI curve peaking in week 52 of 2021 and weeks 12–13 of 2022 [22]. The first peak was mainly due to HRSV, and the second to influenza [23].

SARI are a major cause of hospitalization, particularly during the winter season, and especially among the elderly population and patients with comorbidities [24]. The advent of SARS-CoV-2 emphasized the importance of a broader investigation of respiratory pathogens, both viral and bacterial, in SARI cases. This study aimed at assessing the prevalence and epidemiological aspects of respiratory pathogens in the SARI hospitalized patients in the University Hospital Trust of Siena in Tuscany (Italy) during the season 2023/2024.

## Materials and methods

### *Study population and definition of SARI hospitalization*

Oropharyngeal swabs were collected from enrolled patients in the Emergency Medicine Unit of the University Hospital Trust of Siena for the 2023/2024 season; specifically, sample collection was conducted in the context of the COVIDRIVE project, approved by the Ethics Committee of Liguria N. CET 288/2021 - DB id 11528. Written informed consent was obtained from all patients enrolled.

The study population was composed of hospitalized patients aged ≥ 18 years and meeting the case definition of SARI. A SARI case definition (possible COVID-19 case) was defined as an episode with any of one of this local or systemic symptoms: cough, fever (≥ 38°C), shortness of breath, sudden onset of anosmia, ageusia or dysgeusia. The starting date of symptoms must not exceed 14 days prior to hospital admission. Patients who fulfilled at least two criteria for a SARI case were enrolled in the study.

For each patient, clinical-epidemiological information was obtained by means of a questionnaire. During patient interviews, the following underlying conditions were recorded: asthma, chronic respiratory diseases, cardiovascular diseases, hypertension, chronic liver diseases, chronic kidney diseases, type I or type II diabetes, obesity, cancer (solid and/or hematological cancer), neurological disorders, immunodeficiency or organ transplantation. Vaccination status for COVID-19 and influenza was retrieved from vaccination registry of Local Health Authority of belonging.

### *Laboratory analysis*

Viral RNA was extracted from oropharyngeal swabs using the QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions.

Viral RNA was tested by real-time reverse transcription-polymerase chain reaction (qRT-PCR) using the FTD SARS-CoV-2 Assay kit (Siemens Healthineers GmbH, Erlangen, Germany). Samples showing a cycle threshold (ct) value < 40 were considered as SARS-CoV-2 positive.

Swabs were also tested by qRT-PCR using the FTD Respiratory pathogens 21 kit (Siemens Healthineers GmbH, Erlangen, Germany), a qualitative test used to identify 21 respiratory pathogens: influenza A (IAV); influenza A virus H1N1 (swine strain) (IAV (H1N1) swl), hereafter referred as A(H1N1)pdm09; influenza B virus (IBV); human rhinovirus (HRV); human coronavirus (HCoV) 229E, NL63, HKU1, and OC43; human parainfluenza viruses (HPIVs) 1–4; human metapneumoviruses (HMPVs) A and B; human bocavirus (HBoV); *Mycoplasma pneumoniae (M. pneumoniae);* human respiratory syncytial viruses (HRSVs) A and B; human parechovirus (HPeV); enterovirus (EV); and human adenoviruses (HAdVs).

To obtain information on the infecting HRSV subtype, HRSV-positive swabs were further analyzed with the RealStar RSV RT-PCr kit 3.0 (Altona Diagnostics GmbH, Hamburg, Germany). All qRT-PCR analyses were performed using a Biorad CFX96 Opus thermal cycler.

Swabs positive for SARS-CoV-2 (ct ≤ 30) and/or influenza (ct ≤ 32) were further analysed by Next-Generation sequencing (NGS) with the MiSeq system (Illumina, San Diego, CA, USA).

Library preparation for SARS-CoV-2 sequencing was performed using the COVIDSeq Assay kit (Illumina, San Diego, CA, USA) and Artic V4.1 SARS-CoV-2 primers following the manufacturer's

instructions. The SARS-CoV-2 sequences were assembled with the DRAGEN COVID Lineage software (version 4.0.6).

Library preparation for influenza virus sequencing was performed using the IMAP FLU kit (Illumina, San Diego, CA, USA) following the manufacturer's instructions. The influenza virus sequences were assembled using DRAGEN Targeted Microbial software (version 1.3.1).

*Sequence analysis*

Nucleotide sequences of SARS-CoV-2 and influenza (haemagglutinin (HA) and neuraminidase (NA) genes) were analysed using the Nextclade web application (https://clades.nextstrain.org/) [25]. Molecular phylogeny analyses were conducted using Molecular Evolutionary Genetics Analysis (MEGA) version 11 software with the neighbor-joining model [26]. Reference and/or representative genomes of SARS-CoV-2 and influenza clades were obtained from the Global initiative on sharing all influenza data (GISAID) database (https://gisaid.org/).

*Statistical analysis*

Descriptive statistic (mean, standard deviation and percentage frequencies) and statistical analysis were performed with STATA software (SE/14.0) (StataCorp LLC, College Station, TX, USA). Chi-squared Test was performed to evaluate the association between patients' characteristics and vaccination status (vaccinated for influenza/COVID-19) and number of doses of COVID-19 vaccine received. In addition, logistic regression was performed to evaluate the association between positivity (yes/no) and symptoms: cough (yes/no), followed by shortness of breath (yes/no), by fever ≥ 38°C (yes/no), and Anosmia, ageusia or dysgeusia (yes/no). A confidence level of 95 % ($p < 0.05$) was considered statistically significant.

## Results

*Epidemiology of SARI cases*

From January to June 2024, a total of 138 SARI patients were enrolled in the study, of whom 51 % (N = 70) were female. The mean age of the subjects was 76.7 ± 14.0 years; notably, most of them (82.5 %) were geriatric patients (age ≥65 years). Three subjects (2 %) died during hospitalization.

The 95 % (N = 131) of patients had been vaccinated for COVID-19. The mean doses received by these patients were 3.5 ± 1.3. Of these 131 patients, 11.5 % (N = 15) had received two doses, 36 % (N = 48) three doses, 30 % four doses (N = 39), 17 % (N = 22) five doses and 5 % (N = 7) six doses. No dependence was found between sex ($p = 0.69$) and age ($p = 0.34$) and the number of doses received. As for the influenza vaccine, only 56 % (N = 77) of the 138 patients were vaccinated for the 2023/2024 influenza season. No dependence was found between sex and influenza vaccination ($p = 1.00$), whereas it was observed that most geriatric patients (76 %) had received influenza vaccine, whereas only 32 % of non-geriatric patients were vaccinated ($p = 0.04$).

Most subjects (94 %) had at least one co-morbidity (Table 1), the most frequent of which were cardiovascular diseases (70 %), hypertension (65 %) and chronic respiratory diseases (36 %).

In Table 2 were reported the symptoms observed in the enrolled patients. Most common symptom was cough (99 %), followed by shortness of breath (97 %), by fever (83 %) and anosmia, ageusia or dysgeusia (24 %). No statistically significant difference was found in the distribution of symptoms and age (fever $p = 0.46$; cough $p = 0.42$; shortness of breath $p = 0.32$; anosmia, ageusia or dysgeusia $p = 0.845$) and sex (fever $p = 0.07$; cough $p = 0.99$; shortness of breath $p = 1.00$; anosmia, ageusia or dysgeusia $p = 0.09$). In addition, no significant association was found between positivity to a pathogen and cough ($p = 0.40$), shortness of breath ($p = 0.30$), anosmia, ageusia or dysgeusia ($p = 0.68$); while fever was more frequent in subjects found positive for at least one pathogen than in negative ones ($p = 0.04$). Out of a total of 138 subjects enrolled, 55 (40 %) were SARS-CoV-2 positive cases. Influenza positive cases were 28 (20 %), of whom 23 IAV (including 5 influenza A(H1N1)pdm09 positive cases) and 5 IBV, while there were 18 (13 %) HRSV positive cases. The other pathogens were found to a lesser extent. Results for each respiratory pathogen included in the study are shown in Table 3.

For 9 of the 18 HRSV-positive swabs, it was possible to obtain information on the subtype. Of these, 7 were found to belong to subtype A and 2 to subtype B.

A total of 34 co-infections were found, of which 16 between SARS-CoV-2 and influenza, 9 between SARS-CoV-2 and HRSV, and 4 between influenza and HRSV.

**Table 1**
Co-morbidities in the study population.

| Underlying conditions | N | % |
|---|---|---|
| Cardiovascular diseases | 97 | 70 |
| Hypertension | 90 | 65 |
| Chronic respiratory diseases | 50 | 36 |
| Cancer (solid and/or hematological cancer) | 26 | 19 |
| Type II diabetes | 24 | 17 |
| Neurological disorders | 21 | 15 |
| Asthma | 19 | 14 |
| Chronic kidney diseases | 13 | 9 |
| Obesity | 9 | 6.5 |
| Immunodeficiency or organ transplantation | 5 | 4 |
| Chronic liver diseases | 2 | 1 |
| Type I diabetes | 1 | 1 |

Number of subjects (N) and percentage (%).

**Table 2**
Symptoms in the study population.

| Symptoms | N | % |
|---|---|---|
| Cough | 137 | 99 |
| Shortness of breath | 134 | 97 |
| Fever ≥ 38°C | 114 | 83 |
| Anosmia, ageusia or dysgeusia | 33 | 24 |

Number of subjects (N) and percentage (%).

**Table 3**
Laboratory-confirmed positive-cases for respiratory pathogens included in the study.

| Respiratory pathogen | N | % |
|---|---|---|
| SARS-CoV−2 | 55 | 40 |
| Influenza A | 23* | 17 |
| HRSVs | 18 | 13 |
| Influenza B | 5 | 4 |
| HCoV OC43 | 4 | 3 |
| HRV | 1 | 1 |
| HCoV 229E | 1 | 1 |
| HCoV HKU1 | 1 | 1 |
| HPIVs | 1 | 1 |
| HAdVs | 1 | 1 |
| *M. pneumoniae* | 1 | 1 |
| HMPVs | 0 | 0 |
| HBoV | 0 | 0 |
| HCoV NL63 | 0 | 0 |
| HPeV | 0 | 0 |
| EV | 0 | 0 |

Number (N) and percentage (%) of positive samples for SARS-CoV-2; influenza A; influenza B virus; human rhinovirus (HRV), human coronavirus (HCoV) 229E, NL63, HKU1, and OC43; human parainfluenza viruses (HPIVs); human metapneumoviruses (HMPVs); human bocavirus (HBoV); *Mycoplasma pneumoniae* (*M. pneumoniae*); human respiratory syncytial viruses (HRSVs); human parechovirus (HPeV); enterovirus (EV); and human adenoviruses (HAdVs).

* including 5 cases of influenza A(H1N1)pdm09.

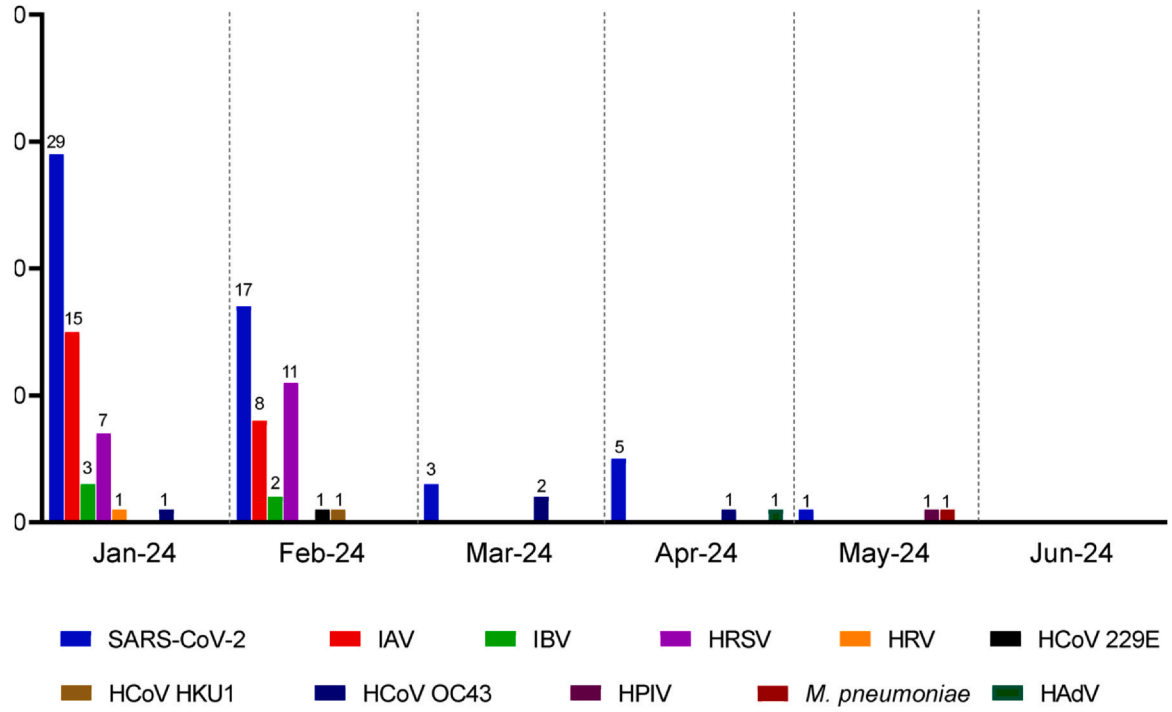


**Fig. 1.** Distribution of confirmed cases of infection during the study period (January - June 2024). Influenza A virus (IAV); influenza B virus (IBV); human respiratory syncytial virus (HRSV); human rhinovirus (HRV), human coronavirus (HCoV) 229E, HKU1 and OC43; human parainfluenza virus (HPIV); *Mycoplasma pneumoniae* (*M. pneumoniae*); human adenovirus (HAdV).

No dependency was found between positivity to influenza and influenza vaccination for the 2023/2024 season, nor between positivity to SARS-CoV-2 and vaccination status/number of doses received for COVID-19.

The subjects enrolled per month were: 47 in January, 38 in February, 21 in March, 20 in April, 11 in May and 1 in June. Fig. 1 shows the distribution of cases of confirmed positivity to at least one pathogen during the period studied. The majority of SARS-CoV-2 and influenza cases were found in January, and the majority of HRSV cases in February. SARS-CoV-2 cases decreased from January to March and increased in April. No cases of influenza and HRSV were detected in March, April and May. Cases of HCoVs infection were found between February and March, while positivity to other pathogens were sporadic. No cases of infection were found in June.

*SARS-CoV-2 sequencing*

Of the 55 SARS-CoV-2 positive swabs, only 4 had a ct value below the defined threshold for sequencing and it was possible to obtain a complete genome sequence. The sequences of these samples were deposited in GISAID (Table S1).

Of these 4 sequences (Figs. 2), 3 were considered variants of interest (VOI): 2 belonged to the Pango JN.1 lineage (clade Nextstrain 24 A), specifically JN.1.1 (sample CD46) and JN.1.21 (sample CD1), with some mutations leading to amino acid substitutions in the receptor binding domain (RBD) of the spike protein compared to the reference for the JN.1 lineage (K403R for sample CD46 and N405D for sample CD1); and 1 belonged to the Pango BA.5.3 lineage (sample CD21, clade Nextstrain 22B), carrying a number of amino acid substitutions in the RBD (F371S, V445H, G446S, N450D, L452N, N460K, N481K, A484K, F486P, R493Q) compared to the reference for BA.5 lineage. The fourth sequence was considered a variant under monitoring and belongs to the Pango HV.1 lineage (EG.5.1, belonging to the XBB family) (sample CD7, clade Nextstrain 23 F). Compared to the reference for XBB.1.5, it has several amino acid substitutions in the RBD (S446N, L452R, F456L).

*Influenza sequencing*

Of the 28 influenza-positive samples, 8 had a ct value below the defined threshold for sequencing and a complete genome sequence was obtained. The sequences of these samples were deposited in GISAID (Table S2).

These 8 samples were all positive for influenza A, of which 3 for A(H1N1)pdm09 and 5 for A(H3N2).

With regard to the samples that tested positive for influenza A(H1N1)pdm09, phylogenetic analysis of the HA gene showed that they belonged to clade 6B.1 A.5a.2a, defined by the amino acid substitutions K54Q, A186T, E224A, R259K, K308R and represented by reference strain A/Sydney/5/2021 (Fig. 3). All viruses belonging to clade 6B.1 A.5a.2a share the amino acid substitution I418V, but subgroups characterized by additional amino acid changes compared to the reference strain were identified, such as: (i) V47I, I96T (sample CD55), (ii) T120A, K169Q (samples CD17, CD44). These amino acid substitutions were also found with respect to vaccine strains A/Victoria/4897/2022 and A/Wisconsin/67/2022 (clade 6B.1 A.5a.2a.1).

Phylogenetic analyses of the HA gene of the samples that tested positive for influenza A(H3N2) virus showed that they belonged to two genetic groups (Fig. 4). Three samples (CD8, CD28 and CD54) belonged to the clade 3 C.2a1b.2a.1a, characterized by the amino acid mutations S198P and K171N and represented by the reference strain A/Cambodia/e0826360/2020. In these samples, a mutation leading to the I160K amino acid substitution compared to the reference strain is also present. These samples also have the amino acid substitutions S156H, N159Y, Q164L, D186R, N190D and T203I compared to the vaccine strains selected for the 2023/2024 season. Two samples (CD11 and CD22) belonged to the clade 3 C.2a1b.2a.3a.1, in which all viruses share the E50K amino acid substitution, with additional amino acid changes (I140K and I223V) also present in the strains selected for the vaccine composition for the 2024/2025 season. A grouping defined by the N122D and K276E mutations was also identified with A/Sydney/878/2023 as the reference strain.

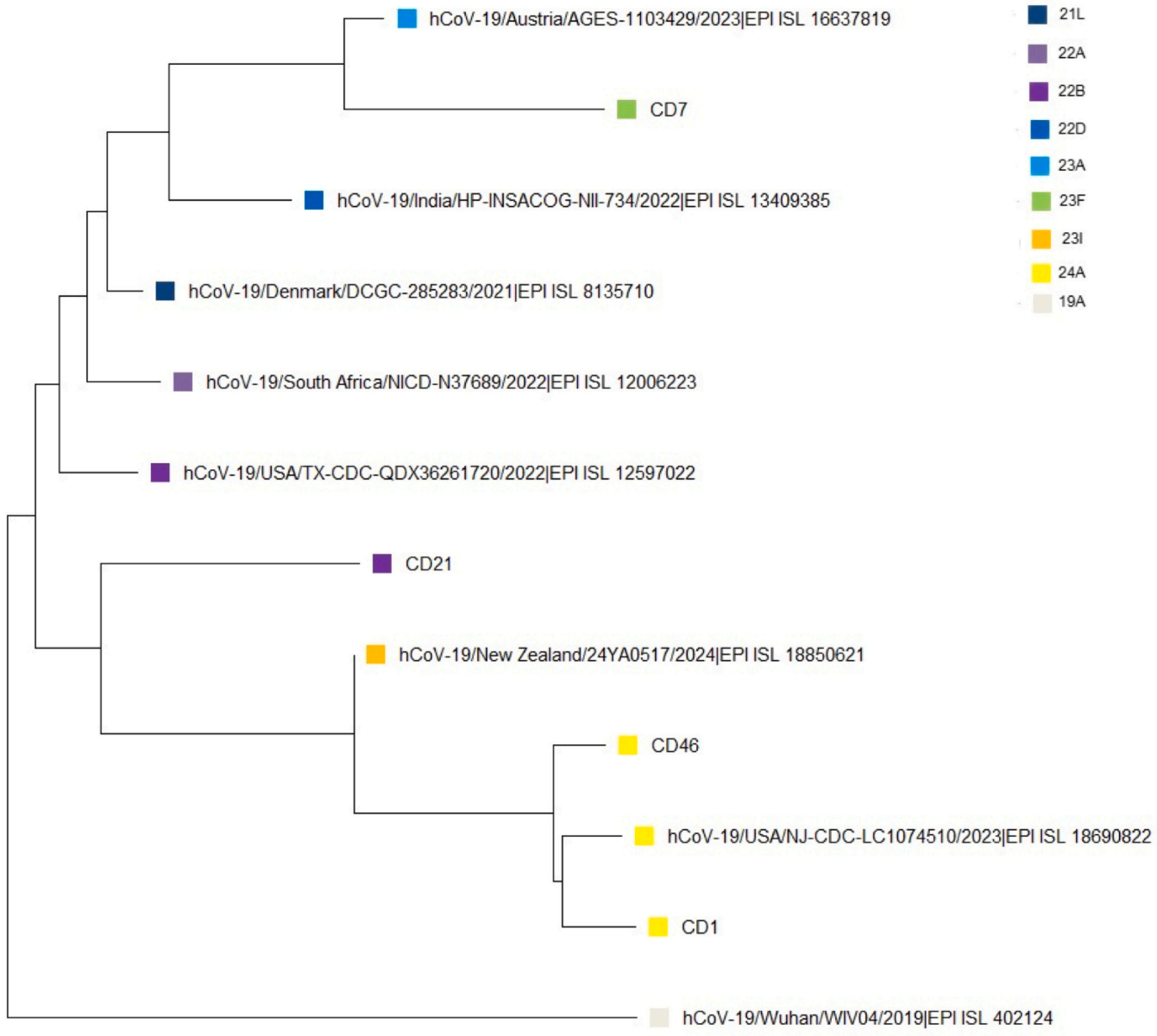


**Fig. 2.** Phylogenetic relationships between the SARS-CoV-2 virus sequences obtained in this study. Some representative strains of the clades they belong to according to the Nextstrain classification (https://nextstrain.org) are reported.

Phylogenetic analysis of the NA gene of both subtypes showed no mutations known to be associated with reduced sensitivity/resistance to oseltamivir or zanamivir.

## Discussion

SARIs represent a major cause of morbidity and mortality globally, especially among the elderly and individuals with comorbidities. The COVID-19 pandemic has contributed to an increase in the number of subjects susceptible to respiratory infectious diseases, both in children and the elderly. During the 2023/2024 season, an increase in viral pneumonias was observed, especially in the elderly, and bacterial pneumonias from streptococcus confirmed in children [27].

This study aimed at evaluating the prevalence and epidemiological aspects of respiratory pathogens in SARI hospitalized patients in the University Hospital Trust in Siena (Tuscany, Italy) during the 2023/2024 season.

From January to June 2024, a total of 138 patients who met the definition of a SARI case, with a mean age of 76.7 years, were enrolled in the study. Most of the subjects had at least one comorbidity, the most frequent of which were cardiovascular and chronic respiratory diseases. Three patients died during hospitalization. Of the total number of subjects enrolled, SARS-CoV-2 was detected in 40 % of cases, followed by influenza in 20 % and HRSV in 13 %. The other pathogens were detected to a lesser extent. In terms of symptoms, cough and respiratory distress were the most frequently reported symptoms, but no association was found between symptoms and specific pathogens, highlighting the importance of using diagnostic tests to distinguish between SARS-CoV-2, influenza, HRSV and other pathogens.

Despite high COVID-19 vaccination rates, breakthrough infections continued to occur, with SARS-CoV-2 cases detected

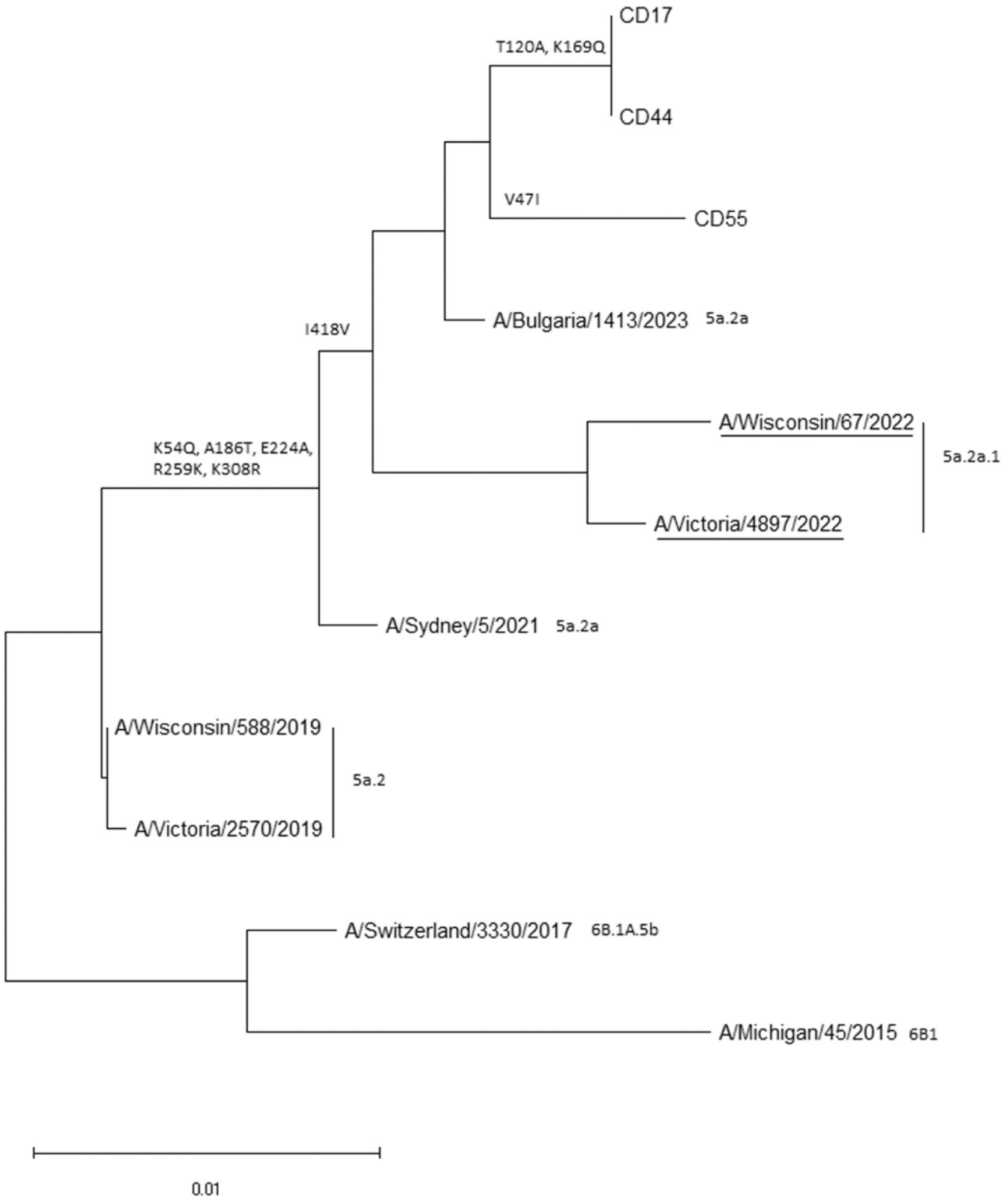


**Fig. 3.** Phylogenetic relationships between the HA gene sequences of the influenza A(H1N1)pdm09 viruses obtained in this study. The reference strains and their clades are shown in the figure; the amino acid substitutions characterizing the clades are shown at the nodes. The vaccine strains for the 2023/2024 and 2024/2025 seasons are underlined.

throughout the season. This highlights the continued circulation of the virus, even in vaccinated populations, although the overall severity of cases was mitigated by vaccination, as evidenced by the low hospital mortality rate. No difference in administration among different demographic groups in the study population were observed, unlike influenza vaccination, for which a gap in vaccination coverage among younger subjects was shown.

Infections with viruses like SARS-CoV-2 often result in asymptomatic cases among very young individuals, likely due to their immune systems' limited inflammatory response. Conversely, elderly individuals tend to exhibit a heightened and dysregulated immune response to viral infections, which can exacerbate disease severity [28]. High serum levels of 25-hydroxyvitamin D are thought to confer protection against viral infections through several

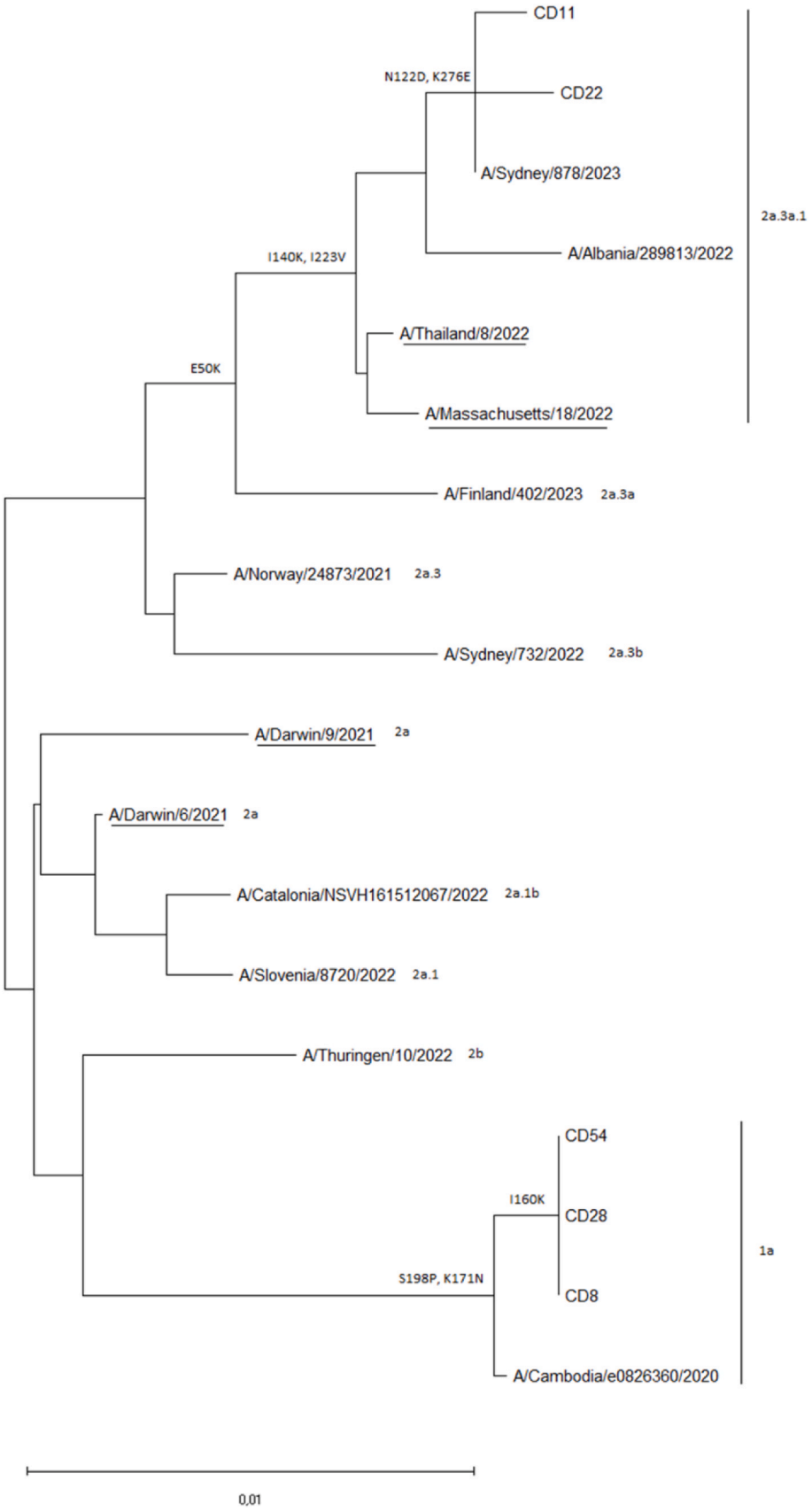


**Fig. 4.** Phylogenetic relationships between the HA gene sequences of the influenza A(H3N2) viruses obtained in this study. The reference strains and their clades are shown in the figure; the amino acid substitutions characterizing the clades are shown at the nodes. The vaccine strains for the 2023/2024 season (A/Darwin/9/2021 and A/Darwin/6/2021) and for the 2024/2025 season (A/Thailand/8/2022 and A/Massachusetts/18/2022) are underlined.

mechanisms, including the upregulation of human cathelicidin, an antimicrobial peptide capable of neutralizing both viruses and bacteria, and by modulating cytokine production, thereby reducing the risk of hyperinflammatory responses such as cytokine storms [29,30].

The seasonal distribution of cases was consistent with the seasonal characteristics of the pathogens, with SARS-CoV-2 and influenza peaking in January. Influenza exhibits a distinct seasonality in temperate climates, with peak incidence during the winter months. This pattern is influenced by a combination of factors, including cold temperatures and low relative humidity, which enhance the stability and prolong the survival of viruses outside the human body. Additionally, cold air can suppress an innate immune response in the nose, reducing the production of extracellular vesicles that normally help fight respiratory viruses [31]. Human behavior, such as spending more time indoors during colder months, and impaired mucociliary clearance due to dry air at low temperatures, also contribute to the increased transmission of influenza and other respiratory viral infections during winter [32–34]. A resurgence of SARS-CoV-2 cases was observed in April, while influenza and HRSV were not detected in the spring months, consistent with their seasonality. These detections were quite in line with national surveillance system, that recorded a constant circulation of different respiratory viruses throughout the winter season. SARS-CoV-2 always circulated constantly until week 52/2023 and then gradually decreased in the following weeks. The incidence rate of influenza reached a value of 18.73 in week 53/2023, one of the highest recorded in recent years. The dominant circulating strain was influenza A/H1N1, concentrating its circulation in the last two months of 2023 and the first months of 2024, and contributing significantly to the incidence peak. HRSV showed a small but steady increase from week to week, eventually dominating into other viruses [35]. A relevant aspect that emerged from the study was the presence of 34 cases of co-infection, which mainly involve SARS-CoV-2 and influenza [36], and which may represent an additional factor of clinical complexity.

Regarding the SARS-CoV-2 strains analysed, the identification of VOI belonging to the JN.1 lineage is consistent with the global spread of this variant and its inclusion in COVID-19 vaccines for the 2024/2025 season [37]. Variants deriving from the BA.5 and XBB.1.5 lineages have also been identified, carrying amino acid substitutions in the RBD known to increase ACE2 receptor binding affinity and/or reduce vaccine efficacy (e.g. F456L, F486P, R493Q) [38,39]. In all these cases, the subject was not vaccinated for the infecting variant.

The phylogenetic analysis of the influenza A(H1N1)pdm09 and A(H3N2) virus strains gave results in line with those reported by the surveillance activities conducted in Italy for the 2023/2024 influenza season [40]. The sequenced influenza A(H1N1)pdm09 virus strains all belong to clade 6B.1a.5a.2a, and have some amino acid substitutions at the HA compared to the vaccine strains for the 2023/2024 season, which were also confirmed for the 2024/2025 season. The analysis of influenza A(H3N2) strains identified two distinct clades (3 C.2a1b.2a.1a and 3 C.2a1b.2a.3a.1), both presenting some amino acid substitutions at the HA compared to the vaccine strains for the 2023/2024 season. This analysis is in line with early vaccine efficacy data for the season, according to which in Canada, during the 2023/2024 season, influenza vaccine efficacy was estimated at 61 % and 49 %, respectively, in preventing influenza related to A(H1N1) and A(H3N2) subtype viruses [41]. In particular, the detection of A(H3N2) strains belonging to clade 3 C.2a1b.2a.3a.1 supports the inclusion of strains belonging to this clade in the recommended vaccine composition for the 2024/2025 season. Phylogenetic analyses of the NA of both subtypes did not reveal the presence of mutations associated with reduced susceptibility or resistance to antivirals.

In conclusion, the 2023/2024 influenza season in Europe and Italy was characterized by the co-circulation of numerous respiratory pathogens, with a high rate of ILI and/or SARI, predominantly associated with influenza and SARS-CoV-2. This study provides a comprehensive overview of the circulation of SARS-CoV-2, influenza, HRSV and other respiratory pathogens during the 2023/2024 season in Italy. A key finding of this study is that several viruses caused severe respiratory tract infections during the winter of 2023/2024, marking a shift from the patterns observed during the initial years of the COVID-19 pandemic. The data obtained support the importance of continuous and integrated monitoring of respiratory viruses, going beyond the traditional focus on influenza viruses alone, to mitigate the clinical and health impact of respiratory infections in the post-COVID-19 era.

## Ethics approval statement

The study was approved by the Ethics Committee of Liguria N. CET 288/2021 - DB id 11528. All experiments were performed in compliance with relevant laws and institutional guidelines and in accordance with the ethical standards of the Declaration of Helsinki.

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## CRediT authorship contribution statement

**Conceptualization:** IM, SM**; Formal Analysis:** SM**; Funding acquisition:** IM**; Investigation:** VS, ACDF, BMB, GM**; Resources:** AC, GB**; Data Curation:** SM, GG; **Project administration:** IM; **Visualization:** IM; **Writing – original draft preparation:** SM, IM**; Writing – review and editing:** VS, BMB, GG, ACDF, GM, EM, GB, AC**.**

## Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: EM is founder and Chief Scientific Officer of VisMederi srl and VisMederi Research srl.

## Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jiph.2025.102905.

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