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RESEARCH ARTICLE

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Molecular epidemiology and disease severity of influenza virus infection in patients with haematological disorders

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Abstract

Influenza virus infection is a common cause of self-limiting respiratory tract infection (RTI), however immunocompromised patients are at an increased risk for a severe course of disease or fatal outcome. We therefore aimed to gain a better understanding of the molecular epidemiology of influenza viruses from patients with haematological disorders and their impact on the clinical course of disease. Molecular analysis using polymerase chain reaction (PCR) of nasopharyngeal swabs was performed for influenza virus in haematological patients at the Heidelberg University Hospital. Clinical data was evaluated to identify associated risk factors. For phylogenetic analysis, the hemagglutinin (HA) gene was sequenced. Out of 159 influenza positive patients, 117 patients developed upper RTI (influenza A: $n = 73$; influenza B: $n = 44$). Lower RTI was observed in $n = 42$ patients (26%), $n = 22/42$ patients developed severe disease and $n = 16/159$ (10.1%) patients died. Risk factors for lower RTI were nosocomial infection ($p = 0.02$), viral shedding for ≥14 days ($p = 0.018$), IgG levels <6 g/dL ($p = 0.046$), bacterial/fungal co-infections ($p < 0.001$). Risk factors for fatal outcome were age ≥65 years ($p = 0.032$), bacterial/fungal ($p \leq 0.001$) co-infections and high viral load ($p = 0.026$). Sequencing of the HA gene ($n = 115$) revealed subtype A(H3N2) ($n = 46$), A(H1N1)pdm09 ($n = 24$), B/Victoria ($n = 34$), B/Yamagata ($n = 11$). There was no correlation between influenza (sub)type and lower RTI. Influenza infection in haematological patients is associated with significant morbidity and mortality, the risk for aggravating co-infections, prolonged viral shedding and nosocomial transmission emphasizing the need for infection control.

KEYWORDS

haematologic disorder, influenza virus, molecular epidemiology, risk factors

1 | INTRODUCTION

Immunocompromised patients with haematological malignancies are at high risk for severe respiratory tract infections caused by influenza virus, leading to increased morbidity and mortality.[1] Whereas influenza virus infections usually present as self-limiting upper respiratory tract infections, it has been shown that immunocompromised patients, especially those post hematopoietic stem cell transplantation, often suffer of more severe life-threatening infections progressing to the lower respiratory tract.[2,3] Influenza viruses are highly contagious and nosocomial transmission is facilitated by prolonged viral shedding in immunocompromised patients resulting in repeated outbreaks in both in- and out-patient wards.[4,5] The persistent threat of outbreaks within hospitals posed by respiratory viruses during the epidemic seasons, as well as the severity of illness, further emphasize the need to protect patients undergoing immunosuppression and to perform systematic screening approaches to prevent nosocomial infection.[6,7]

Influenza viruses utilize antigenic shift and antigenic drift, consequently altering the viral genome frequently and avoiding long-term immunity of the host.[8,9] Vaccination against influenza viruses is recommended in immunocompromised patients, in particular patients who underwent stem cell transplantation.[10] Reduced immune response to vaccination in these patients requires re-evaluation of effectiveness and adjustment of vaccines.[11,12] The significant relevance of respiratory viral infections in immunocompromised patients has been met with increasing acknowledgment and attempts to further improve prevention, hygiene standards and clinical outcome especially considering difficulties in successful development of long-term vaccination.[1,10] Treatment strategies for patients undergoing immunosuppressive therapy include early usage of neuraminidase inhibitors, though their benefit needs to be evaluated individually considering potential side effects of medication.[13]

In our study, we retrospectively investigated patients with influenza virus infection in the haematology and stem cell transplant unit of the University Hospital Heidelberg from 2014 to 2019 and identified risk factors for severe disease and development of LRTI and fatal outcome. Moreover, we performed molecular characterization of influenza viruses investigating their genetic diversity and pattern of cocirculation types and subtypes and their correlation with severe disease.

2 | METHODS

2.1 | Patients and clinical data

We performed a single-center retrospective study with influenza virus positive patients with haematological malignancies of the Heidelberg University Hospital during the winter seasons between 2014 and 2019.

The Heidelberg University Hospital is a tertiary referral center, the department of haematology comprises four inpatient wards for adult patients—two wards for normal and high-dose chemotherapy, one intermediate care unit and one transplant unit—as well as several outpatient clinics. Most of the patients treated suffer from malignant lymphoma, multiple myeloma or leukaemia, each year about 200–250 autologous and 100–120 allogeneic transplantations are performed. As part of the standard operation procedures, from October to March all haematological patients are regularly tested for infection with influenza, parainfluenza and respiratory syncytial virus. Further, symptomatic patients are isolated while awaiting the laboratory result. Infected patients are isolated in single rooms or isolated in cohorts.

Readily available medical records were retrospectively reviewed from all patients to obtain basic characteristics, clinical and laboratory data. Clinical data was analyzed regarding prevalence as well as potential risk factors for severe disease. Patient records and information were anonymized and deidentified before analysis.

2.2 | Definitions

A case of respiratory tract infection with influenza was based on a laboratory-confirmed infection with influenza virus, presenting with or without respiratory symptoms. URTI was defined as laboratory confirmed influenza virus infection without signs of LRTI. LRTI was defined as presence of respiratory symptoms plus radiographic (chest X-ray or chest CT scan) signs of LRTI, severe LRTI was defined as LRTI plus requirement of treatment on intensive care unit or death. Fatal outcome of infection was assessed if death of patient occurred during the course of an influenza infection. Nosocomial infections were defined as virus detection at least 72 h after admission to the ward, the remainders were defined as community-acquired infection.

2.3 | Sample collection and molecular analysis of influenza virus

Nasopharyngeal swabs of all screened inpatients and outpatients who had been positively tested for influenza virus by real time polymerase chain reaction (RT-PCR) were included. For some of the LRTI patients, additional bronchoalveolar lavage (BAL) samples were available.

For molecular analysis, RNA was extracted from respiratory specimens using the QIAamp® viral RNA mini kit (Qiagen) according to the manufacturer's protocol. Reverse transcription, amplification and detection of viral RNA was performed with the RealStar® Influenza RT-PCR kit (altona Diagnostics) on a LightCycler® 480 instrument II (Roche) according to the manufacturer's instructions. This assay distinguished influenza viruses A, A/H1N1 and B. Samples and extracted RNA that tested positive were stored frozen at −20°C at the Clinical Virology Laboratory.

2.4 | Sequencing of influenza virus strains

Classification of influenza subtypes was achieved by sequencing of the viral hemagglutinin (HA) gene. Multisegment one-step reverse transcriptase polymerase chain reaction was used to create and amplify complementary DNA fragments of the viral RNA templates.[14,15] Two primers (Supporting Information: Table 1) target each end of the segment required for transcription and replication. After successful multiplication of the template RNA, second PCR was performed to specifically amplify the HA gene using different sets of primers (Supporting Information: Table 2) resulting in partially overlapping fragments. After purification of PCR products, they were sequenced at GATC Biotech. Nucleotide sequences of influenza A and B strains retrieved in this study were deposited in GenBank (http://www.ncbi.lm.nih.gov) under accession numbers OP647218-OP647262 and OP647136-OP647205.

2.5 | Phylogenetic analysis

The obtained sequencing data was edited with SEQMAN II Software of the Lasergene package (DNASTAR). Due to the length of the HA gene six primers were used for sequencing of influenza A samples and four primers for influenza B, resulting in multiple single sequencing files. These single files were assembled, so a complete sequence of the HA gene could be achieved. MEGA 7.0.26 was used to translate nucleotide to amino acid sequences and create an alignment by using MUSCLE. This alignment allowed for identification of nonsynonymous substitutions relative to A/California/07/2009 (A (H1N1)pdm09) or B/Florida/4/2006 (B/Yamagata); silent nucleotide mutations which did not lead to amino acid substitutions were disregarded. Alignments of the sample collective were compared to reference sequences, which were chosen according to the seasonal WHO reports of the years September 2014–February 2019 and added from WHO samples via online data base Global Initiative on Sharing All Influenza Data (GISAID, gisaid.org; isolate ID numbers in Supporting Information: Table 3). A phylogenetic tree for each influenza subtype containing collected samples as well as reference sequences was constructed using the maximum likelihood method with 1000 bootstraps based on the Jones-Taylor-Thornton model with uniform rates and use of all sites for gap or missing data treatment.

2.6 | Statistical analysis

For statistical analysis Stata Version 15.0 (StataCorp. LP) was used. Demographic and clinical data in our study population was summarized and simple descriptive statistics included medians with interquartile ranges (IQR), and proportions, as appropriate. D'Agostino's K-squared test was applied to test for normal distribution. Univariate group comparisons were performed using χ^2 or Fisher's exact test for categorical variables and by Student's t test or Wilcoxon rank sum test for continuous variables, as appropriate. $p < 0.05$ were considered statistically significant. Risk factors for developing LRTI or for a fatal outcome seen in the univariate comparisons were further analysed using logistic regression models adjusted for age and sex; coefficients (β) and 95% confidence intervals (CI_{95}) were estimated.

2.7 | Ethical approval

The routinely collected samples from patients of the Department for Haematology were stored at the Clinical Virology Laboratory. Permission to use these samples as well as usage of pseudonymized patient information from medical records was obtained from the Ethical Committee of the Heidelberg University Hospital (S-274/2019). Individual written patient consent was waived due to the retrospective and observational character of the study.

3 | RESULTS

3.1 | Patient characteristics and epidemiology

During the seasons 2014–2019, 2782 patients had been tested for influenza virus. In total, 159 (5.7%) patients with pre-existing haematological malignancies have been identified with a positive influenza virus PCR test result and were included for further analysis. Of these 159 patients, 95 patients (59.7%) tested positive for influenza virus type A and 64 patients (40.3%) for influenza virus type B. The distribution of detected influenza type A and B infections per winter season is shown in Figure 1. Key characteristics of these patients are described in Table 1. Univariate analysis of patient characteristics and influenza type showed association of influenza type A with male sex and influenza type B with female sex ($p = 0.004$). The age distribution among patients ranged from 21 to 83 years with an average of 57.1 years (Figure 2). It was similar in both influenza types, the average age for influenza virus type A was 56.6 years (range: 22–83 years) and 57.9 years (range: 21–82 years) for influenza virus type B.

3.2 | Risk factors for severe disease and clinical outcome

Of all 159 cases included in this study, 42 (26.4%) patients categorized as LRTI. Of these, 22 patients (52.4%) were treated on intensive care unit (ICU) or died during influenza infection and therefore categorized as severe LRTI (influenza type A $n = 11/22$ [50.0%]; influenza type B $n = 11/22$ [50.0%]). Risk factor analysis (Table 2) showed significant association with LRTI for IgG levels lower than 6 g/dL ($p = 0.046$), pre-engraftment ($p = 0.027$), with nosocomial origin of infection ($p = 0.008$), and co-infection with other pathogens ($p < 0.001$). Prolonged viral shedding for more than

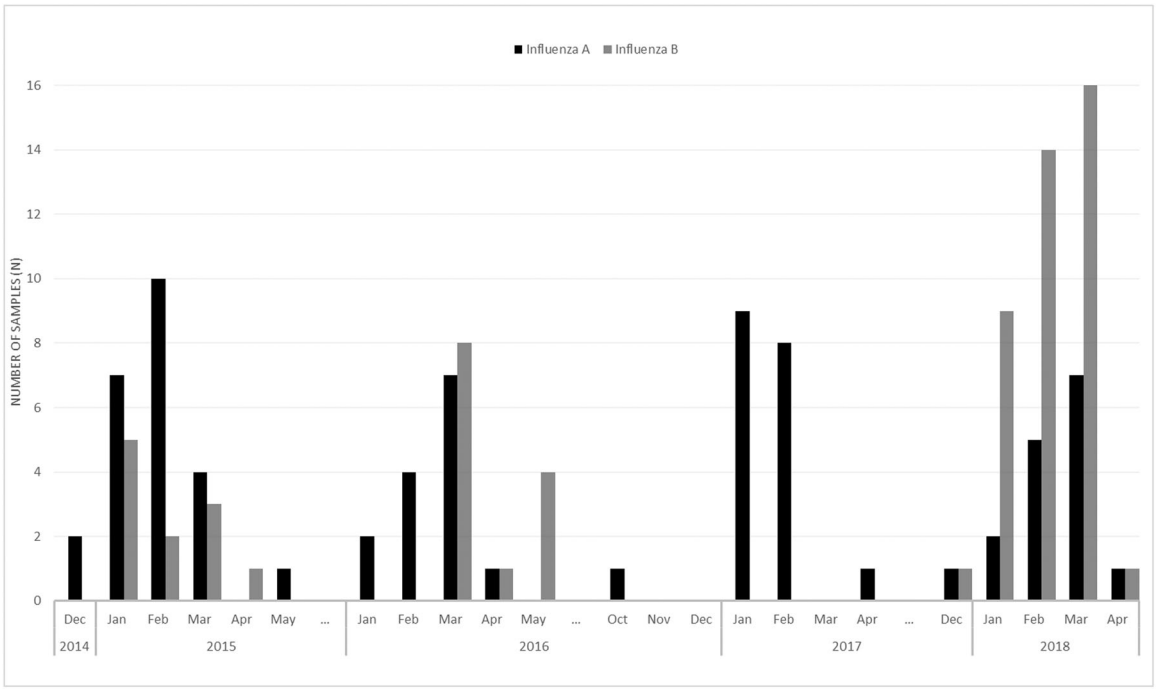

FIGURE 1 Timeline of number of influenza virus infections per month in patients with haematologic malignancies.

14 days ($p = 0.034$) was also associated with LRTI and patients had a significantly longer shedding period with LRTI compared to URTI (median 16.5 vs. 8 days; $p = 0.019$). In multivariate analysis adjusted for age and sex, co-infections ($p < 0.01$; $\beta = 1.4$; $CI_{95}(0.3;2.4)$, Supporting Information: Table 4) remained as significant risk factor for development of LRTI. The most frequently detected co-pathogen was *Enterococcus faecium* ($n = 9/43$), followed by *Aspergillus* ($n = 7/43$), *Escherichia coli* ($n = 5/43$) and *RSV* ($n = 5/43$). Notably, in 9 out of 31 cases of bacterial co-infection, multi-resistant strains were identified. We could not identify any association between a specific influenza virus type and a more severe course of illness.

A fatal outcome was observed in 16 out of 159 influenza infected patients (Table 3), the median survival time was 20.5 days (IQR 7–29 days). Median age of patients with fatal outcome was 65.5 years (IQR 61–68 years), two thirds belonged to the age group of 60 to 69 years. Sex distribution was almost balanced with $n = 9/16$ (56.3%) of patients being female and $n = 7/16$ (43.7%) male. In 75% of patients, active disease was present, which was Non-Hodgkin's lymphoma (NHL) in 38% of cases, multiple myeloma (MM) in 25% of cases, acute myeloid leukemia (AML) and acute lymphoblastic leukemia (ALL) in 13% of cases, smoldering myeloma and chronic lymphocytic leukemia (CLL) in 6% of cases. Influenza virus type A was detected in about half of patients, $n = 4$ were A(H1N1)pdm09 viruses and 3 A(H3N2) viruses. For influenza B, $n = 4$ were B/Yamagata viruses, $n = 2$ B/Victoria viruses and in $n = 3$ samples genotyping failed. All patients classified as a severe LRTI, 94% were hospitalized (median 22 days (IQR 10–35). These patients also received significantly more often IgG therapy ($p = 0.018$) and/or steroids ($p = 0.034$). Transplantation was performed in 56% of cases and bacterial ($n = 8/16$), fungal ($n = 3/16$) or viral ($n = 2/16$) co-pathogens could be detected in 75% of fatal cases. Nosocomial transmission occurred in 31% of cases and mean Cycle threshold (Ct) value was 22 (IQR18.6–24.8). With respect to fatal outcome, age ($p = 0.007$), active disease ($p = 0.016$), IgG levels <6 g/dL ($p = 0.018$) as well as co-infections ($p < 0.001$) showed a significant impact in univariate analysis. Co-infections remained significant in multivariate analysis adjusted for age and sex ($p = 0.03$; $\beta = 2.2$ $CI_{95}(0.3;4.1)$, Supporting Information: Table 5).

3.3 | Viral shedding

Patients with influenza virus infection were followed-up to evaluate duration of viral shedding. Consecutive positive samples in the following weeks were obtained for $n = 38$ patients. Median duration of viral shedding (if ≥5 days) was 12 days (IQR 7–17 days) with 8 days (IQR 7–14 days) in patients with URTI and 16.5 days (IQR 11–19 days) in patients with LRTI. Out of these 38 patients with viral shedding, 21 patients were hospitalized. Prolonged viral shedding for longer than 14 days was observed in $n = 16$ cases of which $n = 4$ presented with prolonged shedding for longer than 30 days. A total of $n = 9$ patients with prolonged viral shedding were presenting with LRTI, and $n = 4$ patients had a fatal outcome.

3.4 | Phylogenetic analysis and influenza subtypes

Successful sequencing of the HA gene of influenza viruses was achieved in a total of 115 samples. For influenza A(H3N2), three

TABLE 1 Key characteristics of haematological patients with influenza infection.

	All *n* = 159	Influenza A *n* = 95	Influenza B *n* = 64	*p* Value[a]
Demographic characteristics				
Age in years, median (IQR)	59 (49–66)	59 (49–65)	61 (50.5–68)	0.34
Female, *n* (%)	70 (44.0)	33 (34.7)	37 (57.8)	**0.004**
Male, *n* (%)	89 (56.0)	62 (65.3)	27 (42.2)	
Underlying disease				
MM, *n* (%)	43 (27.0)	23 (24.2)	20 (31.3)	0.30
HD, *n* (%)	2 (1.3)	0 (0.0)	2 (3.1)	
NHL, *n* (%)	31 (19.5)	19 (20.0)	12 (18.8)	
ALL, *n* (%)	10 (6.3)	5 (5.3)	5 (7.8)	
AML, *n* (%)	37 (23.3)	27 (28.4)	10 (15.6)	
MDS, *n* (%)	12 (7.6)	6 (6.3)	6 (9.4)	
Other, *n* (%)	24 (15.1)	15 (15.8)	9 (14.1)	
Active disease, *n* (%)	72 (45.3)	41 (43.2)	31 (48.4)	0.51
Transplantation, *N* (%)	109 (68.6)	62 (65.3)	47 (73.4)	0.28
Allogenic, *n*/*N* (%)	81/109 (74.3)	46/62 (74.2)	35/47 (74.5)	0.56
Autologous, *n*/*N* (%)	26/109 (23.9)	14/62 (22.6)	12/47 (25.5)	
Allogenic + autologous, *n*/*N* (%)	2/109 (1.8)	2/62 (3.2)	0/47 (0.0)	
Pre-engraftment, *n*/*N* (%)	11/109 (10.1)	6/62 (9.7)	5/47 (10.6)	0.87
Postengraftment, *n*/*N* (%)	98/109 (89.9)	56/62 (90.3)	42/47 (89.4)	
GvHD, *n*/*N* (%)	45/109 (41.3)	27/62 (43.6)	18/47 (38.3)	0.58
Laboratory parameters				
Aplasia, *n* (%)	17 (10.7)	9 (9.5)	8 (12.5)	0.55
IgG < 6 g/dL, *n*/*N* (%)	41/95 (43.2)	22/56 (39.3)	19/39 (48.7)	0.36
Therapeutic interventions				
Ig therapy, *n* (%)	28 (17.6)	17 (17.9)	11 (17.2)	0.91
Steroid therapy, *n* (%)	77 (48.4)	46 (48.4)	31 (48.4)	1.0
IS therapy, *n* (%)	75 (48.1)	43 (46.7)	32 (50.0)	0.69
AVT, *n* (%)	97 (61.0)	58 (61.1)	39 (60.9)	0.99
Coinfections, *N* (%)	43 (27.0)	24 (25.3)	19 (29.7)	0.54
Bacterial, *n*/*N* (%)	19/43 (44.2)	10/24 (41.7)	9/19 (47.4)	0.73
Viral, *n*/*N* (%)	11/43 (25.6)	8/24 (33.3)	3/19 (15.8)	
Fungal, *n*/*N* (%)	5/43 (11.6)	2/24 (8.3)	3/19 (15.8)	
Bacterial + viral, *n*/*N* (%)	4/43 (9.3)	2/24 (8.3)	2/19 (10.5)	
Bacterial + fungal, n/N (%)	3/43 (6.9)	2/24 (8.3)	1/19 (5.3)	
Viral + fungal, *n*/*N* (%)	1/43 (2.3)	0/24 (0.0)	1/19 (5.3)	
Severity of disease				
URTI, *n* (%)	117 (73.6)	73 (76.8)	44 (68.8)	0.26
LRTI, *n* (%)	42 (26.4)	22 (23.2)	20 (31.3)	
Severe LRTI, *n* (%)	22 (13.8)	11 (11.6)	11 (17.2)	0.32

(Continues)

TABLE 1 (Continued)

	All $n = 159$	Influenza A $n = 95$	Influenza B $n = 64$	p Value[a]
Death, *n* (%)	16 (10.1)	7 (7.4)	9 (14.1)	0.17
Hospitalization, *n* (%)	76 (47.8)	44 (46.3)	34 (50.0)	0.65
Duration in days, median (IQR)	14 (6–23)	10.5 (6–19.5)	18 (8–29)	0.11
Origin of infection				
Nosocomial[b], *n* (%)	27 (17.0)	14 (14.7)	13 (20.3)	0.36
Community acquired, *n* (%)	132 (83.0)	81 (85.3)	51 (79.7)	
Viral shedding ≥5 days, *N* (%)	38 (23.9)	24 (25.3)	14 (21.9)	0.62
Duration in days, median (IQR)	12 (7–17)	10 (7–17)	13.5 (10–27)	0.14
Prolonged, >14 days, *n*/*N* (%)	16/38 (42.1)	9/24 (37.5)	7/14 (50.0)	0.45
Prolonged, >30 days, *n*/*N* (%)	4/38 (10.5)	2/24 (8.3)	2/14 (14.3)	0.56

Abbreviations: ALL, acute lymphocytic leukaemia; AML, acute myeloblastic leukaemia; AVT, antiviral therapy; GvHD, graft versus host disease; HD, Hodgkin's disease; IQR, interquartile Range; IS, immunosuppressive; LRTI, lower respiratory tract infection; MDS, myelodysplastic syndrome; MM, multiple myeloma; NHD, non-Hodgkin lymphoma; URTI, upper respiratory tract infection.

[a]Wilcoxon rank sum test for continuous variables, χ^2 test or Fisher's exact test for categorical variables; $p > 0.05$ in bold.

[b]Nosocomial infection = hospitalization took place ≥72 h before first positive test result.

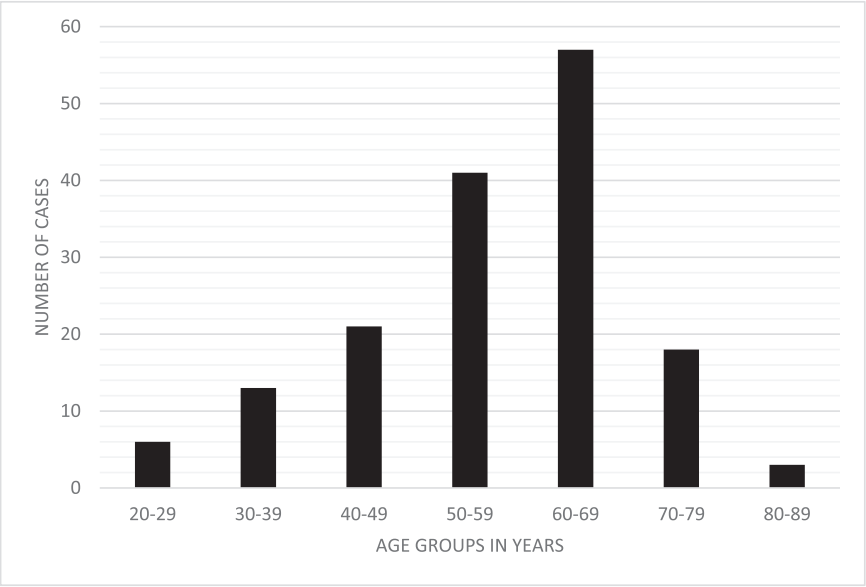

FIGURE 2 Age distribution of haematological patients with influenza virus infection.

genetic clusters were detected. For A(H1N1)pdm09, four clusters could be observed with samples from the seasons 2015/16 and 2018/19. In B/Victoria samples two clusters could be observed and regarding B/Yamagata, two genetic clusters were identified, 22 out of 33 samples from the season 2017/2018 belonging to a certain genetic cluster which is identical to the B/Mauritius/1791/2017 strain. Phylogenetic trees for A(H1N1)pdm09 and B/Yamagata are shown in Figures 3 and 4, respectively. Genetic clusters are marked in the phylogenetic trees. For phylogenetic trees for A/H3N2 and B/Victoria line refer to Supporting Information: Figures 1 and 2.

Clinically, nosocomial transmission was assumed if hospitalization took place ≥72 h before first positive test result, 27 patients (16.9%) met this clinical definition. Analysis of viral strains allowed the identification of clusters with identical amino acid sequence of HA gene and combination of clinical and virological data allows determination of nosocomially transmitted cases with greater confidence. Overall, $n = 11$ phylogenetic clusters could be detected, $n = 11$ patients with nosocomial transmission could be assigned to one of five clusters. Additional $n = 31$ patients that did not clinically qualify as nosocomial could be assigned to one of the clusters. Five patients could possibly be assigned to a cluster but showed an

TABLE 2 Univariate analysis of possible risk factors associated with LRTI.

	URTI *n* = 117	LRTI *n* = 42	*p* Value[a]
Age in years, median (IQR)	58 (48–65)	63 (53–68)	0.07
Age ≥65 years	34 (29.1)	18 (42.9)	0.10
Sex			
Male, *n* (%)	68 (58.1)	21 (50.0)	0.36
Female, *n* (%)	49 (41.9)	21 (50.0)	
Influenza type			
A, *n* (%)	73 (62.4)	22 (52.4)	0.26
B, *n* (%)	44 (37.6)	20 (47.6)	
Underlying disease			
MM, *n* (%)	32 (27.4)	11 (26.2)	0.67
HD, *n* (%)	2 (1.7)	0 (0.0)	
NHL, *n* (%)	20 (17.1)	11 (26.2)	
ALL, *n* (%)	8 (6.8)	2 (4.8)	
AML, *n* (%)	25 (21.4)	12 (28.6)	
MDS, *n* (%)	10 (8.6)	2 (4.8)	
other, *n* (%)	20 (17.1)	4 (9.5)	
Active disease, *n* (%)	50 (42.7)	22 (52.4)	0.28
Controlled disease, *n* (%)	67 (57.3)	20 (47.6)	
Transplantation, *N* (%)	80 (68.4)	29 (69.1)	0.94
Allogenic, *n*/*N* (%)	59/80 (73.8)	22/29 (75.9)	1.0
Autologous, *n*/*N* (%)	19/80 (23.8)	7/29 (24.1)	
Allogenic + autologous, *n*/*N* (%)	2/80 (2.5)	0/29 (0.0)	
Pre-engraftment, *n*/*N* (%)	5/80 (6.3)	6/29 (20.1)	**0.027**
Postengraftment, *n*/*N* (%)	75/80 (93.8)	23/29 (79.3)	
GvHD, *n*/*N* (%)	30/80 (37.5)	15/29 (51.7)	0.18
Laboratory parameters			
Aplasia, *n* (%)	11 (9.4)	6 (14.3)	0.38
IgG <6 g/dL, *n*/*N* (%)	25/68 (36.8)	16/27 (59.3)	**0.046**
Therapeutic interventions			
Ig therapy, *n* (%)	15 (12.8)	13 (31.0)	**0.008**
Steroid therapy, *n* (%)	55 (47.0)	22 (52.4)	0.55
IS therapy, *n* (%)	54 (47.0)	21 (51.2)	0.64
Infections			
Co-infections, *n* (%)	21 (18.0)	22 (52.4)	**<0.001**
Nosocomial infection, *n* (%)	15 (12.8)	12 (28.6)	**0.02**
Viral shedding, *N* (%)	24 (20.5)	14 (33.3)	0.1
Duration in days, median (IQR)	8 (7–14)	16.5 (11–19)	**0.019**

(Continues)

TABLE 2 (Continued)

	URTI n = 117	LRTI n = 42	p Value[a]
Prolonged, >14 days, n/N (%)	7/24 (29.2)	9/14 (64.3)	**0.034**
Prolonged, >30 days, n/N (%)	2/24 (8.3)	2/14 (14.3)	0.56

Abbreviations: ALL, acute lymphocytic leukemia; AML, acute myeloblastic leukemia; GvHD, graft versus host disease; HD, Hodgkin's disease; IQR, interquartile range; IS, immunosuppressive; LRTI, lower respiratory tract infection; MDS, myelodysplastic syndrome; MM, multiple myeloma; NHD, non-Hodgkin lymphoma; URTI, upper respiratory tract infection.

[a]Wilcoxon rank sum test for continuous variables, χ^2 test or Fisher exact test for categorical variables; $p > 0.05$ in bold.

TABLE 3 Characteristics of patients with fatal outcome ($n = 16$).

	Patients with fatal outcome n = 16	Patients *with no* fatal outcome n = 143	p Value[1]
Demographic characteristics			
Age in years, median (IQR)	65.5 (61–68)	58 (48–65)	**0.007**
Age ≥65 years	10 (62.5)	42 (29.4)	**0.007**
Female, n (%)	9 (56.3)	61 (42.7)	0.30
Male, n (%)	7 (43.8)	82 (57.3)	
Influenza subtype			
A/H1N1, n (%)	4 (25.0)	20 (14.0)	0.14
A/H3N2, n (%)	3 (18.8)	68 (47.6)	
B/Yamagata, n (%)	4 (25.0)	26 (18.2)	
B/Victoria, n (%)	2 (12.5)	9 (6.3)	
B/unknown, n (%)	3 (18.8)	20 (14.0)	
Severity of disease			
Severe LRTI, n (%)	16 (100.0)	6 (4.2)	**<0.001**
Hospitalization, n (%)	15 (93.8)	61 (42.7)	**<0.001**
Duration in days, median (IQR)	22 (10–35)	14 (6–23)	0.05
Underlying disease			
MM, n (%)	4 (25.0)	39 (27.3)	0.38
NHL, n (%)	6 (37.5)	25 (17.5)	
AML, n (%)	2 (12.5)	35 (24.5)	
ALL, n (%)	2 (12.5)	8 (5.6)	
CLL, n (%)	1 (6.3)	6 (4.2)	
Smoldering myeloma, n (%)	1 (6.3)	2 (1.4)	
HD, n (%)	0 (0.0)	2 (1.4)	
MDS, n (%)	0 (0.0)	12 (8.4)	
Other, n (%)	0 (0.0)	6 (4.2)	
Active disease, n (%)	12 (75.0)	60 (42.0)	**0.016**
Transplantation, N (%)	9 (56.3)	100 (70.0)	0.26
Allogenic, n/N (%)	7/9 (77.8)	74/100 (74.0)	1.0

TABLE 3 (Continued)

	Patients with fatal outcome *n* = 16	Patients *with no* fatal outcome *n* = 143	*p* Value[1]
Autologous, *n*/*N* (%)	2/9 (22.2)	24/100 (24.0)	
Allogenic and autologous, *n*/*N* (%)	0/9 (0.0)	2/100 (2.0)	
Pre-engraftment, *n*/*N* (%)	0/9 (0.0)	11/100 (11.0)	0.59
Postengraftment, *n*/*N* (%)	9/9 (100.0)	89/100 (89.0)	
GvHD, *n*/*N* (%)	6/9 (66.7)	39/100 (39.0)	0.16
Laboratory parameters			
Aplasia, *n* (%)	1 (6.25)	16 (11.2)	1.0
IgG < 6 g/dL, *n*/*N* (%)	8/10 (80.0)	33/85 (38.8)	**0.018**
Therapeutic interventions			
Ig therapy, *n* (%)	7 (43.8)	21 (14.7)	**0.004**
Steroid therapy, *n* (%)	12 (75.0)	65 (45.5)	**0.034**
IS therapy, *n* (%)	8 (50.0)	67 (47.9)	0.87
Coinfections, *N* (%)	12 (75.0)	31 (21.7)	**<0.001**
Bacterial, *n*/*N* (%)	6/12 (50.0)	13/31 (41.9)	0.35
Viral, *n*/*N* (%)	1/12 (8.3)	10/31 (32.2)	
Fungal, *n*/*N* (%)	2/12 (16.7)	3/31 (9.7)	
Bacterial + viral, *n*/*N* (%)	1/12 (8.3)	3/31 (9.7)	
Bacterial + fungal, *n*/*N* (%)	2/12 (16.7)	1/31 (3.2)	
Viral + fungal, *n*/*N* (%)	0/12 (0.0)	1/31 (3.2)	
Origin of infection			
Nosocomial[2], *n* (%)	5 (31.3)	22 (15.4)	0.11
Community acquired, *n* (%)	11 (68.8)	121 (84.6)	
Viral shedding, *N* (%)	7 (43.8)	31 (21.7)	0.05
Duration in days, median (IQR)	14 (10-39)	11 (7.2)	0.36
Prolonged, >14 days, *n*/*N* (%)	4/7 (57.1)	12/31 (38.7)	0.43
Prolonged, >30 days, *n*/*N* (%)	2/7 (28.6)	2/31 (6.5)	0.15

Abbreviations: ALL, acute lymphocytic leukemia; AML, acute myeloblastic leukemia; GvHD, graft versus host disease; HD, Hodgkin's disease; IQR, interquartile range; LRTI, lower respiratory tract infection; MDS, myelodysplastic syndrome.

[1]Wilcoxon rank sum test for continuous variables, Chi-square test or Fisher exact test for categorical variables; *p* values > 0.05 in bold.

[2]Nosocomial infection = hospitalization took place ≥72 hours prior to first positive test result.

additional mutation. For two samples, no genetically similar cluster could be observed.

4 | DISCUSSION

In adult patients with haematological disorders and stem cell recipients, respiratory viruses are an important cause of life-threatening pneumonia, and are associated with substantial morbidity and mortality. The study cohort included 159 influenza positive patients from the Department of Haematology of the University Hospital Heidelberg from the winter seasons between 2014 and 2019. Associated risk factors for the development of LRTI were nosocomial infection, IgG levels lower than 6 g/dL, prolonged viral shedding for more than 14 days and bacterial, fungal or viral co-infections. No significant association with development of LRTI could be observed for sex, influenza type, underlying disease, transplantation, and engraftment status. Further, the molecular epidemiology of influenza viruses in patients with haematological malignancies was investigated and showed no difference of this patient group

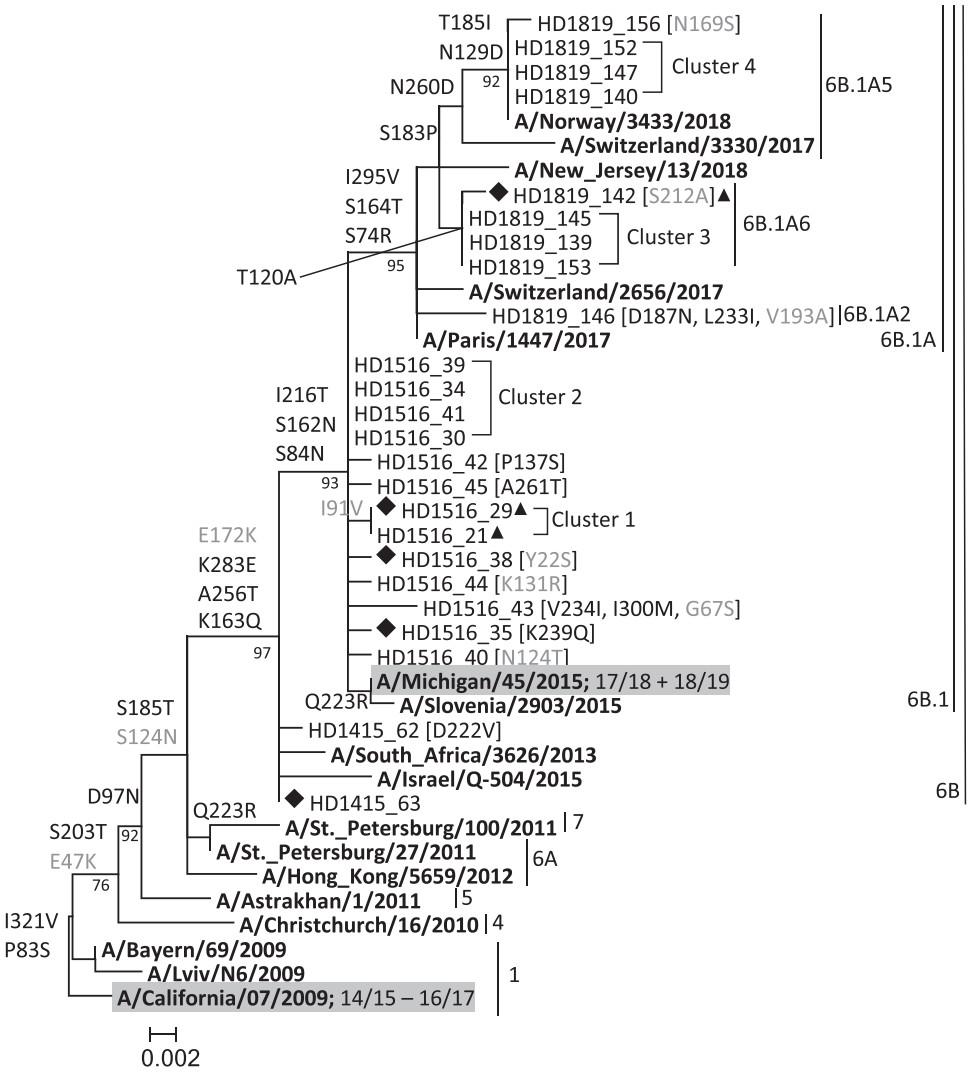

FIGURE 3 Phylogenetic analysis of influenza virus A/H1N1 (A) hemagglutinin gene. Phylogenetic tree from amino acid sequences after removal of signal peptide of influenza virus A strains was constructed with maximum likelihood method with 1000 bootstrap replicates using MEGA 7 software. Influenza virus A strains from this study in Heidelberg, Germany are indicated by the abbreviation "HD" followed by the year of isolation and patient number. Reference strains were retrieved from GISAID and are indicated in bold and by influenza type followed by place of isolation, identification number and year of collection. Assignment to subgroups is indicated by brackets on the right with respective name of subgroup. Vaccine strains are indicated by gray highlighting. Severe cases are marked by "◆" symbol. Nosocomial cases are marked by "▲" symbol. Genetic clusters are shown by brackets on the right. Bootstrap values greater than 70% are indicated at branch nodes. The scale bar represents the number of nucleotide substitutions per site. GISAID, Global Initiative on Sharing All Influenza Data.

regarding influenza type and subtype when compared to the national surveillance data.

Overall, the majority of patients were infected with influenza type A, although the distribution varied between the five winter seasons. In seasons 2016/17 and 2018/19 no influenza type B was detected, whereas in 2017/18 a surge of influenza type B Yamagata line infections could be observed. The latter occurred along with a national increase in excess mortality and was explained by the absence of the Yamagata line in the trivalent vaccine funded by the health system.[16]

Studied haematological patients had an average age of 57.1 years, displaying the range of influenza virus infections in all age groups. An age over 65 was found to be a risk factor for influenza virus infection as well as a more severe course of disease.[17,18] Almost half of patients from this study were older than 60 years and while age ≥65 years was not significantly associated with development of LRTI, 81% of patients who deceased during the course of influenza infection were older than 60 years of age. Furthermore, vaccine efficiency might be lower in older age groups,[19] as well as patients with haematologic malignancies.[20] Unfortunately, no data regarding vaccination was available from this study's patients.

IgG levels lower than 6 g/dL were significantly associated with development of LRTI. Previous studies suggest IgG deficiency to be a risk factor for development of severe influenza infection and Ig therapy to be beneficial for the outcome.[21] Due to inconsistent clinical data on IgG levels under Ig therapy as well as indication of Ig

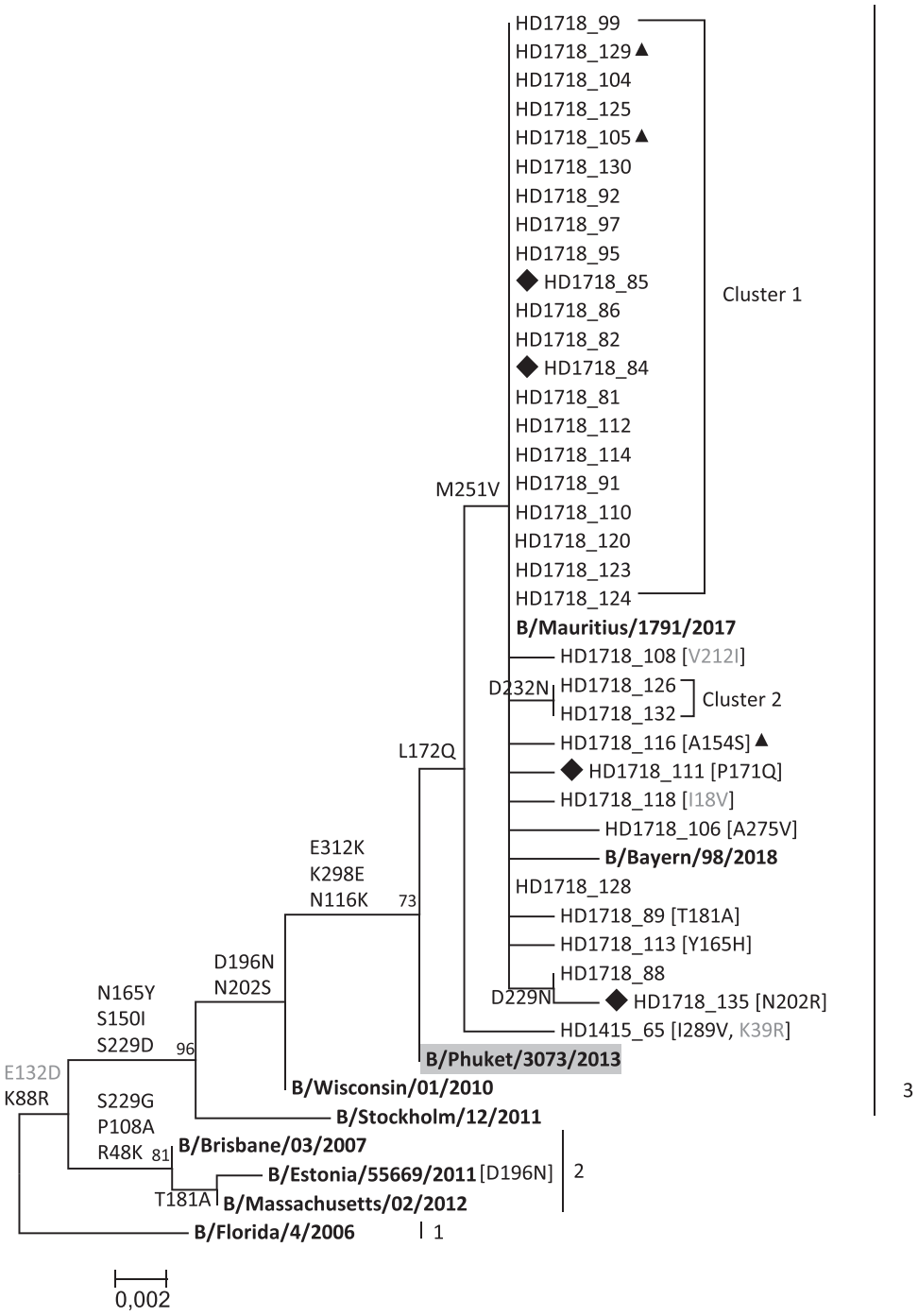

FIGURE 4 Phylogenetic analysis of influenza virus B/Yamagata (B) hemagglutinin gene. Phylogenetic tree from amino acid sequences after removal of signal peptide of influenza virus B strains was constructed with maximum likelihood method with 1000 bootstrap replicates using MEGA 7 software. Influenza virus B strains from this study in Heidelberg, Germany are indicated by the abbreviation "HD" followed by the year of isolation and patient number. Reference strains were retrieved from GISAID and are indicated in bold and by influenza type followed by place of isolation, identification number and year of collection. Assignment to subgroups is indicated by brackets on the right with respective name of subgroup. Vaccine strains are indicated by gray highlighting. Severe cases are marked by "◆" symbol. Nosocomial cases are marked by "▲" symbol. Genetic clusters are shown by brackets on the right. Bootstrap values greater than 70% are indicated at branch nodes. The scale bar represents the number of nucleotide substitutions per site. GISAID, Global Initiative on Sharing All Influenza Data.

therapy, no conclusion about benefits of Ig therapy could be drawn from this patient cohort.

Co-infection has been associated with more severe course of infection including admission to ICU and higher mortality rates, especially bacterial co-infection with *Staphylococcus aureus* or *Streptococcus pneumoniae*.[22–25] We have previously described bacterial co-infection to be an associated risk factor in haematological patients with parainfluenza virus infection.[26] In this study co-infections were detected in 27% of patients with mainly bacterial co-infection. In 9 out of 16 cases with fatal outcome bacterial co-infection could be observed. In total, 15 different bacteria could be identified with most being typical nosocomial pathogens, for example, *E. faecium*, *E. coli*, *S. aureus*, or *Pseudomonas aeruginosa*. Although the origin of co-infections in our study group is not clearly hospital-associated, the high incidence in hospitals as well as the rise of nosocomial pathogens in general make it seem more likely that they are of nosocomial origin.[27] Noticeably 9 of 31 bacterial co-infections were multiresistant strains. Emergence of multiresistant pathogens in hospitals is a common problem and greater susceptibility of this particular immune deficient group as well as high frequency of influenza infection with bacterial superinfection is giving cause for concern.[28]

Further, RSV was the most frequent viral co-pathogen. Viral co-infection of influenza and RSV has been shown to increase likelihood of hospital admission but not necessarily more severe outcome in form of admission to ICU or death.[29] In this study's observations only one of five RSV coinfected cases was hospitalized, though this patient also showed *Aspergillus* co-infection. None of the fatal cases presented with viral respiratory co-pathogens. *Aspergillus* co-infection has been detected in seven cases of which four were fatal, implying great severity of *Aspergillus* co-infection. This is in line with data describing common occurrence and significance of aspergillosis.[30–32] While the rate of LRTI development is similar to previous reports, the mortality rate in patients with LRTI is 38% and therefore considerably higher than 11%–33%, as reported previously for influenza virus infection as well as higher than we have observed for other viral infections, such as parainfluenza virus or respiratory syncytial virus in patients with haematological malignancies.[1,26,33] This might be due a monocentric study as well as inclusion of the season of 2017/18 which was registered as the strongest in Germany since beginning of the influenza surveillance program from the RKI in 2001 and also presented most fatal cases in this study with 6 out of 57 cases. Still, it clearly indicates the need for more alertness and improvement of infection control. Regarding patients with fatal outcome, 5 out of 16 patients presented with Ct values lower than 20 indicating higher viral loads, which might be associated with more severe outcome. Influenza type and subtype in cases with fatal outcome were mostly in line with the seasonal predominant subtypes, so no specific susceptibility for this patient collective seems likely.

Investigation of viral disease transmission is of importance to be able to diminish infection spread and outbreaks, especially with immunocompromised patients on haematologic wards. While viral clearance in the respiratory tract usually occurs after 3 to 5 days, a prolonged period for influenza viruses as well as other viral pathogens has been observed in immunocompromised patients in previous studies by our group as well as others.[4,34] In this study cohort viral shedding for 7 days or less has been observed in 60% of patients with consecutive testing. 13 patients presented with prolonged viral shedding for more than 14 days, of these 23 were cases of URTI and 13 of LRTI. Regarding influenza type, median duration of viral shedding was shorter for influenza type A than B (7 ± 8.5 vs. 13.5 ± 22.0 days), which was in line with an earlier publication.[34] The importance and incidence of prolonged viral shedding particularly regarding the risk for more severe disease outcome demonstrate the necessity of thorough hygiene policies, especially concerning isolation measures to prevent spread of infection.

Molecular characterization of respiratory viruses has the potential to aid in the identification of infection chains. The phylogenetic analysis revealed that the prevalence of influenza subtypes matched with those reported from the German National Influenza Surveillance. In total, 11 phylogenetic clusters with identical sequence could be observed. 73% of B/Yamagata samples and 82% of B/Victoria belonged to one cluster and cluster sizes were relatively large with 67% and 55% of samples belonging to a single cluster. This further strengthens previous reports of influenza type B viruses lower mutation rate. Especially influenza type A(H3N2) observations over years suggest an increase of the virus's potential to mutate with no samples showing identical sequence in the season of 2018/19. The high mutation rate of influenza A compared to B and especially A (H3N2) has been documented before.[35,36] Clinically, nosocomial origin of infection was assumed when hospitalization took place ≥72 h before first positive test result, this was the case in 27 patients, 11 patients could certainly be assigned to a phylogenetic cluster of identical HA gene sequences. Comparison of cases with URTI to LRTI (n = 15/117 vs. n = 12/42 cases) displayed significant association (p = 0.02) of nosocomial origin and LRTI. This is in line with literature's association of nosocomial transmission and severity of disease.[37]

This study is subject to several limitations. This is a monocentric study, data might not be nationwide representative. Only clinical and virological data that had been already available could be analysed in our retrospective study. In addition, a mixed cohort of in-patients and outpatients were included in this study, and nontransplant as well as transplant patients were included. Further, some of the community-acquired cases had been previously treated as outpatients, therefore nosocomial infection was a possibility. Since data was analysed retrospectively, no control group nor information of infection with other viruses were available for further comparisons.

5 | CONCLUSION

Evaluation of possible risk factors confirm particularly bacterial co-infection to be significantly associated with development of LRTI in influenza infected patients with haematological disorders. The high

number of multiresistant bacteria is also of great concern considering less effective therapeutic options and higher susceptibility of this patient group. Bacterial superinfections remain a great cause of worry, demonstrating the importance of strict hospital hygiene, as well as vaccination. Low IgG levels and therefore IgG deficiency are likely to be a risk factor for development of severe influenza infection, thus Ig therapy should be taken into consideration as a therapeutic and maybe even preventive option. With viral shedding lasting longer than 7 days in 40% and prolonged viral shedding for more than 14 days being an associated risk factor to development of LRTI, it indicates the necessity of more thorough hygiene policies, especially concerning isolation measures to prevent spread of infection. This is further emphasized by analysis of phylogenetic clusters and nosocomial transmission. There is a considerable mortality associated with haematological patients suffering from influenza virus infection with a lethality rate underlining the relevance of risk factors and possible course of actions regarding prevention.

AUTHOR CONTRIBUTIONS

Conception or design of the work: Julia Tabatabai, Paul Silan Silan Ünal. *Data collection*: Silan Ünal, Nicola Giesen, Julia Tabatabai, Marianne Wedde, Ralf Dürrwald Paul Silan. *Data analysis and interpretation*: Silan Ünal, Nicola Giesen, Julia Tabatabai, Paul Silan. *Drafting the article*: Silan Ünal, Paul Silan, Julia Tabatabai. *Critical revision of the article*: Silan Ünal, Paul Silan, Nicola Giesen, Marianne Wedde, Ralf Dürrwald, Julia Tabatabai. *Final approval of the version to be published*: Silan Ünal, Paul Silan, Nicola Giesen, Marianne Wedde, Ralf Dürrwald, Julia Tabatabai.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available in GenBank at http://www.ncbi.lm.nih.gov, reference number OP647218-OP647262, and OP647136-OP647205.

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