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DOCTORAL THESIS

Evaluation of the particularities of the host-pathogen interaction in influenza virus infection

ABSTRACT

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PhD thesis abstract

"Evaluation of the particularities of the host-pathogen interaction in influenza virus infection"

The thesis is divided into 2 parts: a general part, consisting of 4 chapters, in which the current state of knowledge is presented through a synthesis of the most recent studies published on the topics addressed in the thesis; and a part of personal contributions, in which the results of the 4 studies carried out during the doctoral studies are presented.

GENERAL PART

Chapter 1. Characteristics of the pathogen - influenza viruses

This chapter covers concepts related to the structure of influenza viruses, with a focus on influenza A and B viruses. We outlined the segmented RNA nature of the virus, with 8 segments, each encoding structural and non-structural proteins [1, 2]. We also summarized recent studies on the role of the surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA), in viral replication and reassortment [3, 4]. In section 1.2, we reviewed the epidemiologic characteristics of influenza viruses, focusing on disease burden, seasonality, natural reservoirs, genetic variability, and molecular epidemiology.

Chapter 2. Characteristics of the human host

In this chapter, we explained that the human host response to influenza virus infection is not always the most effective, depending on certain characteristics such as age, associated comorbidities, genetic features or immune memory determined by previous antigen exposure through vaccination or prior infection. We identified the risk groups for severe influenza according to the European Centre for Disease Prevention and Control (ECDC) [5], World Health Organization (WHO) [6] and the U.S Centers for Disease Control and Prevention (CDC) [7]. In particular, we analyzed the particularities seen in pregnant women, newborn, toddlers, preschoolers, elderly adults, the obese patient, the immunosuppressed patient, and the patient with multiple chronic diseases. Finally, we discussed the susceptibility and progression to severe influenza through the involvement of genetic factors. [8].

Chapter 3. Clinical expression of influenza virus infection in humans

Chapter 3 outlines the algorithm for establishing the diagnosis of influenza based on clinical, anamnestic, and epidemiologic criteria and the use of various laboratory techniques to identify influenza viruses. The third subsection (3.3) summarizes the complications that can occur in patients with influenza as a result of different mechanisms such as: the direct action of influenza viruses on the affected organ/tissue, a disproportionate systemic inflammatory response of the host, decompensation of the patient's chronic disease, viral/bacterial superinfections. At the same time, we highlighted complications that may occur later, after recovery from influenza.

Chapter 4. Pathogenesis of the interaction with influenza viruses

This chapter presents a novel approach to characterizing and understanding the interaction between influenza viruses and the human host. We have shown that during influenza infection, humans develop innate and adaptive immune responses that aim to eliminate the infection, but are unique to each individual. The ultimate goal is to eliminate the pathogen or to at least reduce the negative impact of the infection. [9]. However, a host that is unable to mitigate the effects of the influenza virus or to tolerate the consequences of the immune response to infection becomes prone to develop an unfavorable course of disease [9]. The mechanisms of both non-specific and specific immune responses are summarized in an original figure created with publishing license using the software BioRender.com. In subsection 4.2, we highlighted the specific interactions at the molecular level of influenza viruses with pregnant women, infants and toddlers, older adults, and patients with obesity. All data are also summarized in original figures created with publishing license using the software BioRender.com.

PERSONAL CONTRIBUTIONS

Chapter 5. Working hypothesis and general objectives

The main objectives of this thesis were to assess the dynamics of the local epidemiology of influenza viruses and to characterize the particularities of their interaction with the human host during infection.

The objectives of the PhD thesis derived from the stated aims were:

- O1. Characterization of seasonal influenza circulation by virus type and subtype/lineage and host characteristics in patients requiring hospitalization;

- O2. Characterization of the clinical picture of influenza infection according to virus types and subtypes/lineages and patient characteristics;
- O3. Assessing the clinical course of patients hospitalized for influenza by age, comorbidities, and co-infections;
- O4. Identification, characterization, and stratification of risk factors for adverse outcomes in patients hospitalized for influenza;
- O5. Characterization of the molecular epidemiology of influenza viruses;
- O6. Identification of associations between the genetic evolution of influenza viruses and host characteristics;
- O7. Assessment of the impact of the COVID-19 pandemic on the epidemiology of influenza viruses and changes in the epidemiology of respiratory viruses;
- O8. Assessing the acceptability of influenza vaccination as a measure to modulate the host-pathogen interaction.

Chapter 6. General methodology of the doctoral studies

In this chapter, we explained that the objectives are achieved through four studies: the first, presented in chapter 7, addresses objectives 1, 2, 3, and 4; the second, presented in chapter 8, addresses objectives 5 and 6; the third, presented in chapter 9, addresses objective 7; and the fourth, presented in chapter 10, addresses objective 8.

We also presented the international grants financed through competition that supported the studies described in chapters 7 and 8: the Global Influenza Hospital Surveillance Network (GIHSN) prospective active epidemiologic surveillance study and the Development of Robust and Innovative Vaccine Effectiveness (DRIVE).

In this chapter we have also detailed the statistical analysis tests that were used in the analysis of the data in this thesis.

Chapter 7. Epidemiological characteristics of influenza viruses and characteristics of patients hospitalized with influenza in the 2018-2023 seasons

This chapter presents the results of the first study; it is structured into introduction, methods, results, discussion and conclusions.

Methods

We conducted an observational epidemiological active prospective surveillance study of hospitalized influenza for 5 consecutive seasons (2018/19 - 2022/23), through the participation of the National Institute for Infectious Diseases "Prof. Dr. Matei Balș" (NIID) in annual projects funded by two large international consortia: GIHSN [10] and DRIVE [11].

This study was conducted annually for 5 consecutive seasons from November to April each year; this timespan is included in the official national surveillance period for acute respiratory infections such as: influenza-like illness (ILI) and severe acute respiratory infections (SARI).

The patients included in the study met all the inclusion criteria defined below: patients hospitalized in the National Institute for Infectious Diseases "Prof. Dr. Matei Balș" for ILI/SARI, with onset of symptoms in the previous 7 days, who signed the informed consent and for whom it was possible to collect a respiratory sample in the first 48 hours after admission.

The following study procedures are described: case identification, informed consent signing and eligibility verification, completion of a standardized medical questionnaire, collection of a respiratory sample, identification of influenza viruses by RT-PCR, and monitoring patient evolution.

Results - Epidemiological characteristics of the influenza viruses circulating in the timespan 2018 through 2023

Among patients hospitalized for ILI/SARI during the five studied seasons, we identified an overall influenza positivity rate of 41.9%. The circulation of influenza viruses was uneven across the 5 seasons analyzed, with different influenza positivity rates: 41.2% in the 2018/19 season, 49.5% in the 2019/20 season, 0% in the 2020/21 season, 31.7% in the 2021/22 season, and 50.6% in the 2022/23 season. (Figure 7.1).

Influenza A viruses predominated in 79.3% of cases. According to viral subtypes/lineages, the distribution of influenza cases was as follows: 38.2% A/H1, 33.6% A/H3, 7.3% untyped A, 20.5% B/Victoria and 0.4% untyped B. The median age of influenza-positive patients was significantly younger than that of influenza-negative patients (7.6 years vs. 13.9 years, $p<0.001$). Influenza B viruses were more frequently isolated from children ($p<0.001$, OR=3.2), especially from schoolchildren and adolescents ($p<0.001$).

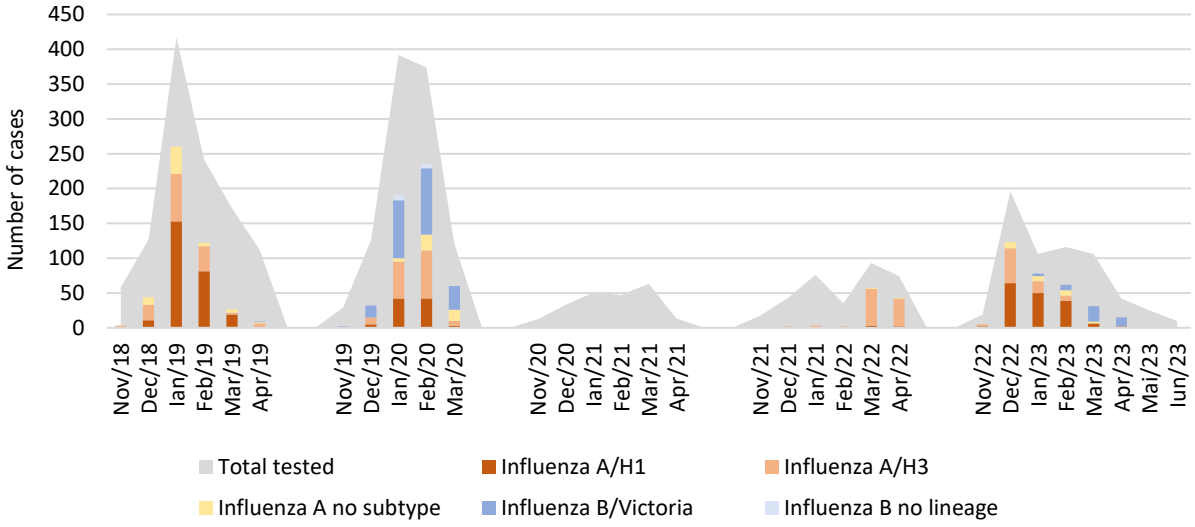

Figure 7.1. Monthly distribution of positive influenza cases by subtype over the 5 seasons

Results – Clinical and evolutionary comparisons between positive and negative cases

In terms of clinical presentation, we found that the most common symptoms in patients with influenza were fever (96.1%) and cough (91.0%). In addition, we stratified the risk of symptoms as follows: patients with influenza had a 4.4-fold increased risk of fever ($p<0.001$), a 2.5-fold increased risk of nasal congestion ($p<0.001$), a 1.8-fold increased risk of cough ($p<0.001$), a 1.5-fold increased risk of myalgia ($p<0.001$), a 1.3-fold increased risk of odynophagia ($p=0.002$), and a 1.2-fold increased risk of headache ($p=0.005$). In contrast, the presence of dyspnea (OR=0.6, $p<0.001$), diarrhea (OR=0.5, $p<0.001$), anosmia (OR=0.2, $p=0.001$) and chest tightness (OR=0.2, $p=0.001$) were negatively associated with influenza. (Figure 7.2).

Multivariate analysis of symptom combinations showed that fever (OR=4.4) remained the best predictor of influenza during the cold season, followed by the combination of fever and cough (OR=2.7, $p<0.001$), fever and nasal congestion (OR=2.2, $p<0.001$), and fever, cough, and nasal congestion (OR=2.2, $p<0.001$).

In addition, we calculated the sensitivity of symptom associations in identifying positive influenza cases and showed that the association of fever, cough, nasal congestion, and myalgia had the best sensitivity, of 80.9% ($p<0.001$).

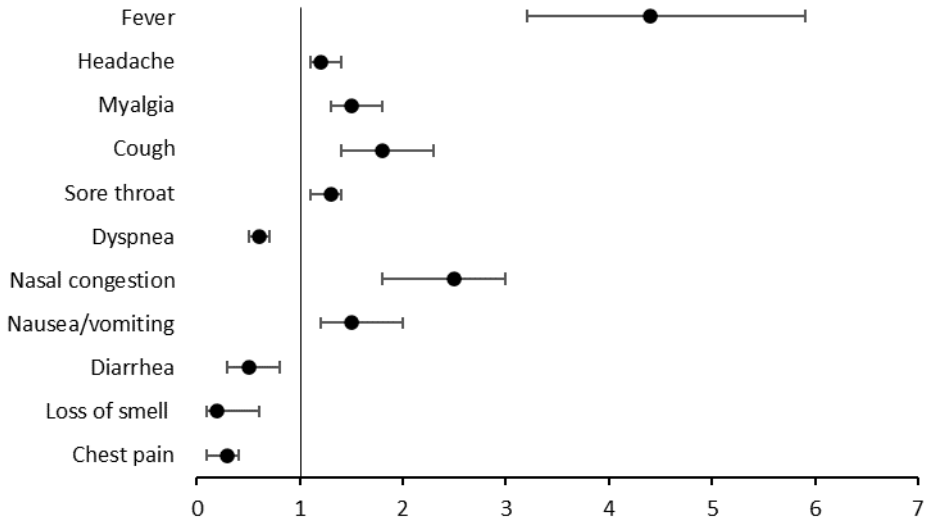

Figure 7.2. Clinical manifestations associated with influenza

Results - Clinical and evolutionary features of the host with influenza

The median length of hospital stay for all influenza patients in the study was 4 days and was significantly longer for infants (5 days, $p<0.001$) and the elderly (8 days, $p<0.001$). After excluding all risk factors related to age, chronic diseases, co-infections and all cases with associated complications, the median length of hospital stay for patients with uncomplicated influenza was reduced to 2 days ($p<0.001$) (Figure 7.3).

We identified the impact of risk factors on the length of hospitalization of influenza patients. Thus, the median length of hospital stay was 6 days for patients with at least 1 chronic disease ($p<0.001$) and 8 days for patients with at least 3 chronic diseases ($p<0.001$). Specifically, patients with obesity had a median length of stay of 6 days ($p<0.001$), and patients with cardiovascular disease, diabetes, and neoplastic disease had a median length of stay of 7 days ($p<0.001$ for each). Patients with pneumonia required 5.5 days of hospitalization ($p<0.001$), and the development of respiratory failure and ICU admission prolonged hospitalization up to 8 days ($p<0.001$, for each).

The incidence of respiratory failure in patients with influenza was 9.9%. Elderly patients (8.9-fold increased risk, $p<0.001$) and the presence of chronic diseases (7.0-fold increased risk in the presence of at least 1 chronic disease and 11.5-fold increased risk in the presence of at least 3 chronic diseases, $p<0.001$ for each) were the best predictors of progression to respiratory failure. Among the chronic diseases, patients who were positive for influenza and COPD

(OR=13.4, p<0.001), cardiovascular disease (OR=9.2, p<0.001), obesity (OR=6.4, p<0.001), neoplastic disease (OR=6.3, p<0.001), or chronic kidney disease (OR=7.9, p<0.001) were most likely to develop respiratory failure. (Figure 7.4).

We identified that late presentation to hospital, more than 4 days after symptom onset, increased the risk of respiratory failure 2.4-fold (p<0.001), highlighting the need for early initiation of antiviral treatment to reduce the risk of adverse outcomes.

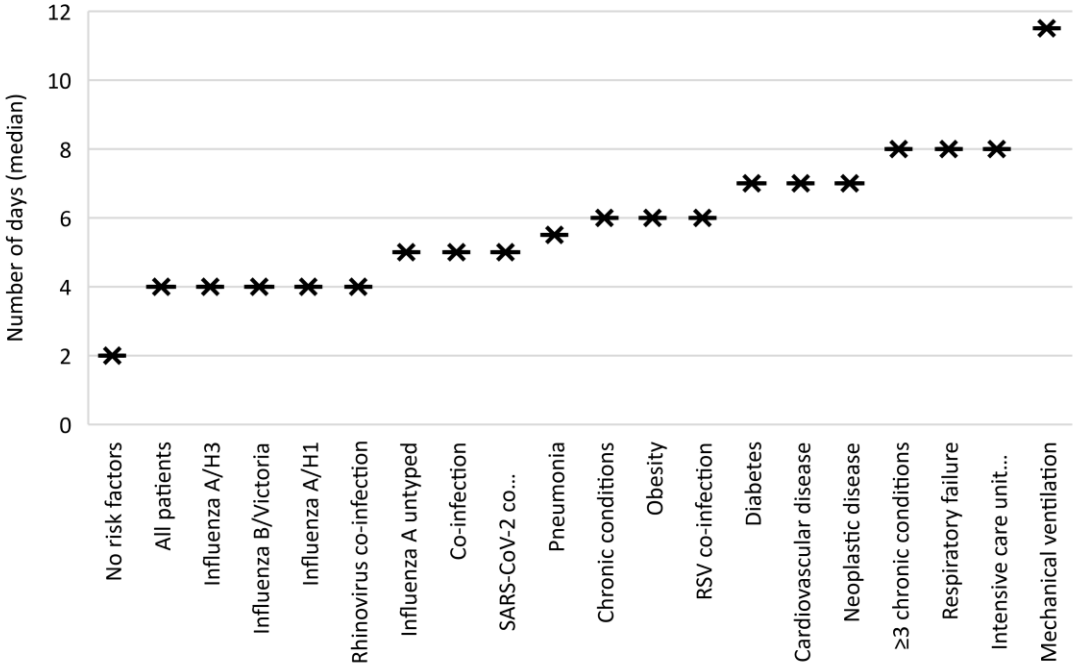

Figure 7.3. Median length of hospitalization in relation to risk factors

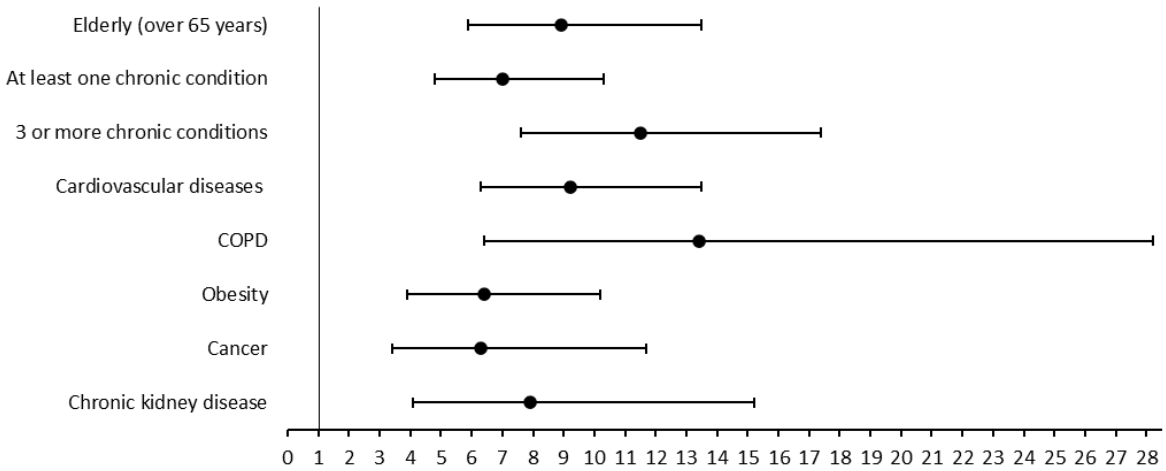

Figure 7.4. Risk of respiratory failure associated with host characteristics

Chapter 8. Genetic particularities of influenza viruses in relation to host particularities (2019-2023 seasons)

This chapter presents the results of the second study; it is structured into introduction, methods, results, discussion and conclusions.

Methods

We performed genetic sequencing of influenza viruses isolated from patients hospitalized in NIID. This analysis is based on positive influenza samples identified in the active prospective surveillance observational epidemiologic study presented in Chapter 7. The period analyzed covers the 2019/20, 2021/22 and 2022/23 seasons.

All patient characteristics were obtained from the administered questionnaires and the clinical monitoring, as described in Chapter 7.

After selection, samples were prepared for sequencing by nucleic acid purification. Next generation sequencing was performed using Illumina technology (San Diego, California, USA). The resulting nucleotide sequences were processed using BLAST (Basic Local Alignment Search Tool) and Geneious Prime.

Phylogenetic analysis was performed on the coding segment of the HA reference sequence using Multiple Alignment using Fast Fourier Transform (MAFFT) [12] and FastTree programs implemented in Mega 6.4 [13] and FigTree v.1.4.4.

Analysis of mutations associated with oseltamivir resistance in the NA gene was performed using FluServer, implemented in the GISAID platform.

Vaccine efficacy (E) was estimated using the $P_{epitope}$ model.

Results - Molecular characteristics of influenza A/H1 viruses and host specificities

In the 2019/20 season, 18 A/H1 isolates were sequenced, all belonging to clade 6b1.A. An average of 9 amino acid mutations per HA sequence was found in the strains in the study, including the Sa, Sb and Ca2 antigenic sites. Vaccine efficacy was reduced to E=44%.

In the 2021/22 season only 3 A/H1 samples were sequenced, all classified in clade 6B.1A.5a.1. Two of the 3 sequences were identical and epidemiologically linked. In relation to the strain included in the seasonal vaccine we identified a number of mutations, one of these mutations was located in the Sa antigenic site (N130K) and two others in the Sb antigenic site (D187A and A189E); the latter reduced the efficacy of the seasonal vaccine to E=33%.

In the 2022/23 season a total of 121 A/H1 samples were sequenced, belonging to clades 6B.1A.5a.2a and 6B.1A.5a.2a.1. We identified mutations in the antigenic sites that could affect

seasonal vaccine efficacy. All sequences showed 2 mutations in the Sb antigenic site (A186T and Q189E), which could reduce the efficacy of the seasonal vaccine to E=33%. Four of the sequenced viruses also had 2 mutations in the Ca2 antigenic site (P137S, K142R); the latter had the potential to reduce vaccine efficacy to E=23%; all four sequences belonged to clade 6B.1A.5a.2a.1.

Results - Molecular characteristics of influenza A/H3 viruses and host specificities

In the 2019/20 season, 20 A/H3 samples were sequenced and grouped into 2 clades, 3C.3a and 3C.2a1b, the latter clade being characteristic of Southern Hemisphere strains. In the sequences of clade 3C.2a1b (4/20), we identified the T135K substitution associated with the loss of an N-glycosylation site in the A antigenic site, which improves the immune response by making this antigenic site more accessible to neutralizing antibodies. Sequences in clade 3C.3a showed few mutations, whereas sequences in clade 3C.2a1b showed an increased number of mutations compared to the vaccine strain, reducing vaccine efficacy to E=12%.

In the 2021/22 season, 61 A/H3 samples were sequenced with a heterogeneous distribution in 6 clades showing an important evolution of A/H3 viruses in this season: 3C.2a1b.2a.1, 3C.2a1b.2a.2, 3C.2a1b.2a.3, 3C.2a1b.2a.3a, 3C.2a1b.2 a.3b, 3C.2 a1b.2a.2c, 3C.2a1b.2a.2. The antigenic site B was dominant, all sequenced viruses showed 6 mutations compared to the sequence of the virus included in the seasonal vaccine, resulting in complete loss of vaccine efficacy.

In season 2022/23, 63 A/H3 samples were sequenced, grouped into 3 clades 3C.2a1b.2a.2b, 3C.2a1b.2a.2a.3a.1 and 3C.2a1b.2a.2a.1b, different from the previous season. We identified between 6 and 10 non-synonymous mutations per sequence, as well as mutations in all antigenic sites (A-E), with a reduction in vaccine efficacy between E=18.5% and 43%. We identified cases with specific mutations causing a reduction in vaccine efficacy up to E=8% in isolates belonging to patients with obesity and cardiovascular disease.

Results - Molecular characteristics of influenza B viruses and host specificities

In the 2019/20 season, all 29 sequenced influenza B virus samples belonged to clade V1A.3. Most mutations identified were from adult patient samples, with the majority located in the 120-loop antigenic site, resulting in a decrease in vaccine efficacy to E=77%.

During the 2022/23 season, 37 influenza B virus samples were sequenced, all belonging to clade V1A.3a.2, but with numerous mutations in the antigenic and non-antigenic sites, resulting in a distribution into numerous sub-clades. Mutations identified in the 190-helix

antigenic site reduced vaccine efficacy to E=58.64%, and those identified in the 120-loop antigenic site reduced vaccine efficacy to a lesser extent to E=64%.

Results - Molecular analysis of the neuraminidase gene and incidence of oseltamivir resistance mutations

We identified 4 cases with oseltamivir resistance mutations: 3 in influenza A/H1N1 viruses (2.4% resistance rate, 2 H275Y mutations, 1 I223V mutation) and 1 case in influenza A/H3N2 viruses (0.8% resistance rate, I222V mutation). All were isolated in patients with risk factors.

Chapter 9. Impact of the COVID-19 pandemic on influenza virus epidemiology

This chapter presents the results of the third study; it is structured into introduction, methods, results, discussion and conclusions. This study was divided into two sub-studies.

Sub-study 1 methods

We conducted an analysis of multiplex RT-PCR data on the epidemiology of respiratory viruses other than influenza viruses among patients hospitalized with ILI/SARI. This study is based on the prospectively obtained data and the same methodology described previously in Chapter 7.

Sub-study 2 methods

We conducted a cross-sectional study based on clinical practice data on influenza re-emergence among children evaluated in the Emergency Department (ED).

The study period was March 1-28, 2022, and the study population included children (patients up to 18 years of age) who presented to the ED with ILI. All pediatric patients with at least one ILI symptom who were tested for influenza or SARS-CoV-2 infection were consecutively enrolled in the study.

Results - Impact of the COVID-19 pandemic on the circulation of respiratory viruses among hospitalized patients

Besides influenza, RSV circulation was most affected by the COVID-19 pandemic; after the onset of the pandemic, circulation levels were significantly lower compared to the pre-pandemic period (Figure 9.1). RSV and human coronaviruses were isolated from the adult and elderly hospitalized ILI/SARI population in significantly higher proportions than the other respiratory viruses ($p<0.001$). Rhinovirus circulation was least affected by the COVID-19 pandemic. Following SARS-CoV-2 (OR=14.6), RSV was the next virus to be associated

(OR=1.1) with the risk of developing acute respiratory failure in ILI/SARI hospitalized patients, while adenovirus infection was associated with the lowest risk (OR=0.09).

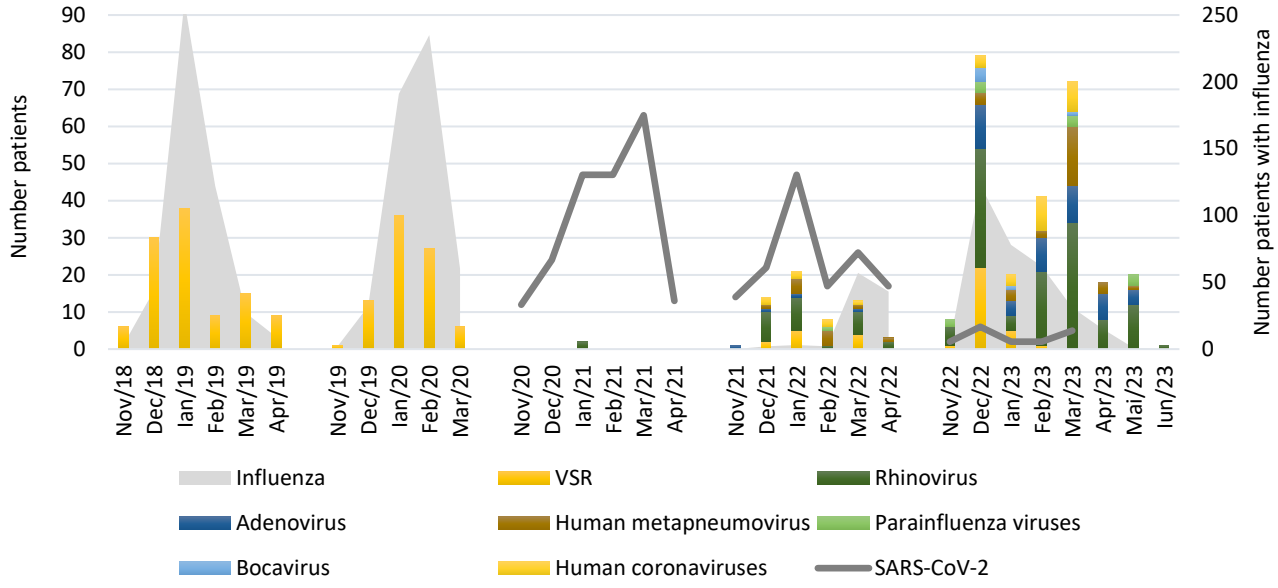

Figure 9.1. Distribution of cases by seasons and months according to virus type

Results - Re-emergence of influenza in the 2021-2022 season - analysis of data from real-world practice

The lifting of pandemic-related restrictive measures and the emergence of the Omicron variant led to the re-emergence of influenza viruses with a 33.5% positivity rate in pediatric patients evaluated in the emergency department. The re-emerging influenza viruses circulated in parallel with a continued but relatively low activity of SARS-CoV-2, for which we identified a 7.5% positivity rate during the same period. Only 10 (2.4%) of the children with influenza evaluated in the ED required hospitalization.

Chapter 10. Understanding vaccination-based interventions for modulating host-pathogen interactions through vaccination

This chapter presents the results of the fourth study; it is structured into introduction, methods, results, discussion and conclusions.

Methods

We conducted a cross-sectional study to assess parents' knowledge, perceptions and attitudes about childhood vaccination and vaccine-preventable diseases.

The questionnaire was administered online in August 2021. For the integrated data analysis, we calculated a knowledge score and a knowledge level based on the questionnaire responses.

In the present analysis we focused on data directly relevant to the studied disease, looking at parents' knowledge, perceptions and attitudes about influenza and influenza vaccination.

Results

We found an influenza vaccination rate of 17.7% among the children of the responding parents. We showed that there was a positive association between the knowledge score and knowledge level about vaccination and vaccine-preventable diseases and the influenza vaccination rate ($p<0.001$).

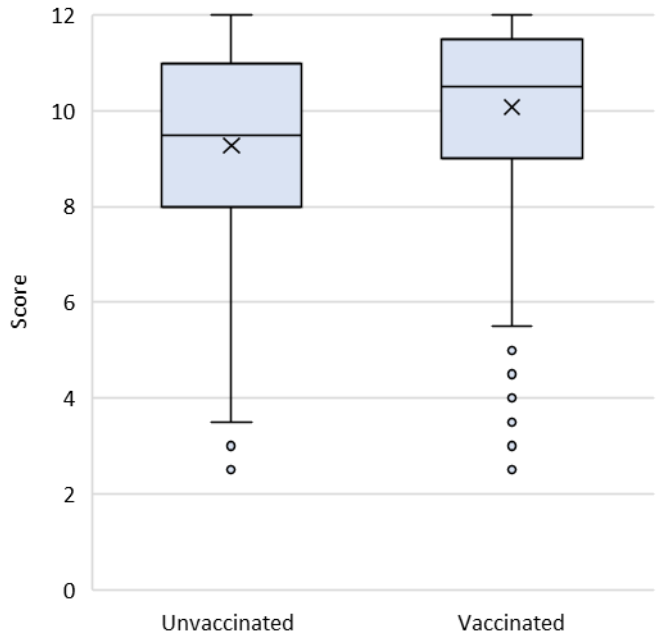

Figure 10.1. Knowledge score in relation to influenza vaccination options

We quantified the reasons for refusal/hesitancy regarding influenza vaccination: 18.4% feared adverse effects, 8.5% were worried about high cost, 8.4% underestimated the impact of influenza infection, 3.6% had received pediatrician recommendation against vaccination. We found that 38.8% of parents would like to have the influenza vaccine included in the free vaccination program. We also showed that fear of a possible severe influenza outbreak increased the likelihood of influenza vaccination by a factor of 2.8.

Final conclusions

Influenza viruses have an impressive genetic diversity due to natural reservoirs identified in birds and mammals, with the ability to cross the species barrier and adapt to humans, potentially leading to outbreaks, epidemics or pandemics. Monitoring the circulation of influenza viruses has become a rule, in order to prevent epidemic outbreaks and to select the most suitable strains for the composition of influenza vaccines to ensure their high efficacy. As the evolution of influenza viruses is closely linked to host and environmental factors, emphasis is placed on surveillance covering as many geographical areas as possible and identifying local trends in virus evolution. Studying the interaction with the human host is an essential factor for understanding genomic changes of influenza viruses, as the immune system exerts a selective pressure, while viruses in turn find immune evasion mechanisms through genetic reassortment, the emergence of antiviral resistance mutations or mutations that lead to reduced vaccine efficacy.

Through our results, we have extensively characterized the circulation of influenza viruses in relation to patient demographics and we have identified specific patterns of viral subtypes/lines.

We have quantified for the first time the likelihood and sensitivity of signs and symptoms from the ILI/SARI definitions to predict positive influenza diagnosis among hospitalized patients.

We have identified the impact that risk factors have on patients with influenza in terms of length of hospitalization and risk of progression to acute respiratory failure. These results are particularly important for quantifying the risks of patients with influenza shortly after admission in order to make the best therapeutic decisions to improve prognosis.

For the first time in Romania, we performed a molecular analysis of influenza virus isolates, with identification of mutations, assessment of the vaccine efficacy and evaluation of associations with host specificities. Understanding the evolution of influenza viruses in relation to the characteristics of the population in which they circulate is essential for targeted and personalized interventions in the prevention and control of future influenza epidemics and pandemics.

In addition, we have performed a screening analysis for mutations in the NA gene associated with oseltamivir resistance to assess the local situation.

In Chapter 9, we evaluated in an original way (double analysis of hospitalized patients, and patients evaluated in the Emergency Department) the impact that the COVID-19 pandemic had on the circulation of influenza viruses, but also on other respiratory viruses, as a result of an expanded capacity to test ILI/SARI patients by multiplex RT-PCR from respiratory samples. We have shown that the COVID-19 pandemic has altered the epidemiology of respiratory viruses in ways that are still incompletely understood, but that the re-emergence of influenza viruses has occurred at similar or even higher attack rates compared to pre-pandemic seasons.

In the last chapter, we attempted to understand the reasons for low influenza vaccination rates in an original population-based study. The results can form the basis of public health policies to increase vaccination rates, the single most effective mechanism for positively modulating the interaction between influenza viruses and the human host.

The Ph.D. thesis is based on documentation from 345 bibliographic sources, articles indexed in Web of Science journals, of which 301 were published in the last 5 years (2019-2023).

The thesis also contains 53 original figures and tables, and the results are supported by 8 appendices detailing part of the results.

From the PhD studies, 4 original articles have already been published, all in journals indexed in Web of Science, with a cumulated impact factor of **16.457.** In addition, 16 abstracts have been published following the presentation of data from the dissertation at national and international congresses and conferences.

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List of published articles from studies carried out in the PhD thesis:

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   → Chapter 7, chapter 8, Appendix 2, pages: 66-70, 75-95,112-129, 198-199
2. Drăgănescu AC, **Miron VD***, Streinu-Cercel A, Florea D, Vlaicu O, Bilaşco A, Oţelea D, Luminos ML, Piţigoi D, Streinu-Cercel A, Săndulescu O. *Circulation of influenza A viruses among patients hospitalized for severe acute respiratory infection in a tertiary care hospital in Romania in the 2018/19 season: Results from an observational descriptive epidemiological study*. Medicine (Baltimore). 2021;100(52):e28460. PMID: 34967388; WOS:000736149900039; ISSN: 1536-5964; **IF(2021): 1.817**; original article, *autor corespondent.
   http://doi.org/10.1097/MD.0000000000028460
   → Chapter 7, Appendix 2, pages: 65-66, 75-95, 198-199
3. **Miron VD**, Bar G, Filimon C, Craiu M. *From COVID-19 to Influenza-Real-Life Clinical Practice in a Pediatric Hospital*. Diagnostics (Basel). 2022;12(5):1208. PMID: 35626363; WOS:000803645600001; ISSN: 2075-4418; **IF(2022): 3.6**; original article, prim-autor.
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   → Chapter 9, pages: 139-140, 145-147
4. **Miron VD**, Toma AR, Filimon C, Bar G, Craiu M. *Optional Vaccines in Children-Knowledge, Attitudes, and Practices in Romanian Parents*. Vaccines (Basel). 2022;10(3):404. PMID: 35335036; WOS:000774394700001; doi: 10.3390/vaccines10030404; ISSN: 2076-393X; **IF(2022): 7.8**; original article, prim-autor.
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   → Chapter 10, pages: 152-157