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Evaluation of nasal levels of interferon and clinical severity of influenza in children

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ABSTRACT

Background: Experimental data show that type I interferon has a key role in innate immune response against influenza infection.
Objective: We compared nasal levels of interferon-α2 and β among inpatients and outpatients with influenza.
Study design: Children younger than 5 years of age with influenza-like illness seeking care at the emergency department within the first 72 h of disease onset were prospectively included. Clinical and demographic data and secretions through nasal wash were obtained. Influenza infection was assessed through reverse-transcription polymerase chain reaction and nasal levels of interferon-α2 and β were measured by enzyme-linked immunosorbent assay. All patients followed until the end of the disease.
Results: One hundred patients were included, of which 24 had confirmed influenza infection, and 5 of them were hospitalized. Subtypes A (H3N2) and B were confirmed in 10 and 14 patients, respectively. Seventy-six patients without influenza, including 48% of outpatients, were recruited as controls. All hospitalized patients were significantly younger regardless of influenza status (age < 6 months in 59% vs. 23.2%, $p < 0.001$). All other data were similar among the groups. Comparing median levels of interferon-α2 among children with influenza, levels were significantly higher in outpatients than in hospitalized patients and were 263.2 pg/mL (25–75 interquartile range: 58.3–634) and detectable in only one patient (90 pg/mL), respectively. The levels of interferon-α2 in controls and those of interferon-β in all groups were not detected.
Conclusions: Higher levels of interferon-α2 in patients with less severe influenza reinforce experimental evidence about the protective role of interferon-α2 against influenza infection.

1. Background

Influenza virus infection is a major cause of morbidity and mortality in children [1,2]. Despite extensive research, influenza infection remains a challenge in clinical practice. Although the clinical course is self-limited in most children, a subset of patients will have respiratory failure and die [3–5]. Clinical characteristics of the influenza virus are difficult to distinguish from those of other respiratory viruses [6–11]. Risk factors for the severe clinical course are comorbidities and young age, but complications may occur in children without these conditions [12–17]. Additionally, antiviral treatments modestly affect the clinical course [18,19]. The majority of disease in humans are associated to types A and B. Influenza A is related to a greater burden of disease and has an important antigenic variability due to combination of

Abbreviations: DFA, direct immunofluorescence assay; IFN, Interferon; IFN-I, type I interferon; ILI, influenza-like illness; NS1, non-structural protein 1; RSV, respiratory syncytial virus

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haemagglutinin and neuraminidase subtypes, leading to seasonal epidemics and pandemics [20].

The knowledge of complex influenza pathogenesis is evolving and may offer scientific background for better clinical management. Extensive data from experimental studies, mainly with influenza type A, highlight early innate immunity as a key factor for host protection [21–24]. The induction of interferon (IFN) release, in its types I (α and β) (IFN-I), type II(γ) and type III(λ) has a central role in the response to many viruses, including influenza [21,25,26]. Interferons are both a paracrine and an autocrine agent and acts in the activation of immune system, stimulating the antigen presentation and signaling other cells about ongoing infection [25,26]. Different types and subtypes (of IFN-α) are released by distinct cells according to each viral infection, reflecting specialized functions of immunity [26]. Although conclusions vary, most data from mice and human cells suggest that deficient production of IFN-I, or its inhibition through viral non-structural protein 1 (NS1), increases the severity of influenza infection [22,22,23,24]. Studies in humans' cells have shown that groups at higher risk for complications, such as elderly and pregnant women, have a deficient IFN-I production [27,28]. Breastfeeding was related to a higher IFN-I production during influenza infection, which could be one of protective mechanisms of human milk, especially IFN-α [29]. Thus, the characterization of IFN-I production in both outpatients and hospitalized children with influenza infection, besides contributing to the knowledge about the role of IFN-I in the immunity against this virus, can also give insight into new prognostic tests and treatments.

2. Objective

We compared the nasal levels of IFN-α2 and IFN-β among hospitalized and non-hospitalized children with influenza.

3. Study design

Study population and design: A cohort of children younger than 5 years of age were recruited at the emergency department of Hospital São Lucas da PUCRS in Porto Alegre, Brazil with influenza-like illness (ILI) and/or signs/symptoms of acute lower respiratory tract infection within the first 72 h of disease. The case definition of ILI was fever (axillary temperature > 37.8 °C), sore throat, and/or a cough without a known diagnosis other than influenza. In children younger than two years, ILI was defined as fever associated with running nose and cough. Acute lower respiratory tract infection was defined as a cough, wheezing, retractions, and/or crackles with or without fever. Exclusion criteria were the use of oseltamivir during enrollment and sample collection and absence of nasal secretion enough for viruses and IFN analysis. The need for hospitalization for use of supplemental oxygen was the severity-related outcome. Clinical and demographical data included age, race, comorbidities, average family income, and breastfeeding status. Children were considered breastfed if receiving breast milk at least once daily.

Nasal secretions were obtained to identify viruses and detect IFN; 1 mL of saline solution was instilled in each nostril followed by vacuum aspiration of secretions. Hospitalized patients were followed until discharge. For outpatients, parents or caregivers were contacted by phone from 10 to 15 days after inclusion and further need of hospitalization was checked. The patients included with the same clinical criteria mentioned above whose influenza infection was ruled out were used as controls.

Virus detection: Nasal secretions were aliquoted and stored up to 24 h at 4 °C. RNA was extracted using PureLink® viral DNA/RNA mini kit (Invitrogen) following the manufacturer's instructions, and RNA was quantified using Qubit™ (Invitrogen). cDNA was synthesized using Superscript III (Invitrogen). The quality of the cDNA for each patient was tested by amplifying the b-actin endogenous gene using specific primers and probes for TaqMan Assay (HuACTB; Applied Biosystems by Life Technologies) and TaqMan Master Mix (Applied Biosystems) on StepOne™ (real-time polymerase chain reaction; Applied Biosystems). PCR conditions were recommended by the TaqMan Master Mix protocol. The samples whose gene transcription could not be detected were excluded. Specific primers and probes were used for amplification of influenza A (Forward 5′-GACCRATCCTGTCACCTCTGAC-3′, Reverse 5′-CGGTGCTCTTGACCAAATTGG-3′ and Probe FAM 5′-TGCAGTCCTC GCTCACTGGGCACG–3′BHQ1). If influenza A was detected through this first test, subtypes were tested through the following primers: influenza A subtype H1N1 (Forward 5′-AACTACTACTGGACTCTGCTGGAA-3′, Reverse 5′-CCATTGGTGCATTTGAGGTGATG-3′ and Probe FAM TGAYCCA AAGCCTCTACTCAGTGCGAAAGC BHQ1), and influenza A subtype H3N2 (Forward 5′-AAGCATTCCYAATGACAAACC-3′, Reverse 5′-ATTGCRCCRAATATGCCTCTAGT-3′ and Probe FAM CAGGATCAC ATATGGGSCCTGTCCCAG BHQ1). Influenza B was tested through primer: Forward 5′-TCCTCAACTCACTCTTCGAGCG–3′, Reverse 5′CGG TGCTC TTGACCAAATTGG –3′ and Probe FAM CCAATTCGAGCAGCT GAAACTGCGGTG BHQ1. Direct immunofluorescence assay (DFA) in nasal secretions was performed in hospitalized patients as described previously [30].

Immunoassay for IFN detection: IFN-α2 (a mixture of IFN-α2a and α2b) and IFN-β levels were measured by sandwich Enzyme-linked Immunosorbent Assay (PBL Interferon Source, Piscataway, NJ, USA) in supernatants of nasal washes after homogenization and centrifugation, following the manufacturer's instructions. The IFN-α2 ELISA assay ranges of detection used were 12.5–500 pg/mL (High Sensitivity) and 156–5000 pg/mL (Extended Range). For the IFN-β ELISA, the range of detection was 50–4000 pg/mL. Briefly, standards and samples (100 µL) were added to the plates and incubated for 1 h. Then, the plates were washed once with wash buffer and the diluted antibody (100 µL) was added to the wells. After 1 h of incubation, the plates were washed three times with wash buffer, and horseradish peroxidase solution (100 µL) was added to the wells and incubated for 1 h. The plates were then washed four times with wash buffer, and tetramethyl benzidine substrate (100 µL) was added to each well and incubated for 15 min in the dark. The stop solution (100 µL) was added to each well and the absorbance was measured at 450 nm in a microplate reader (EZ Read 400, Biochrom) immediately.

Sample size and statistical analysis: Sample size calculation was performed according to a pilot unpublished study carried out by the associated researchers from Argentina. As mean difference in nasal levels of IFN-α in mild patients compared with severe patients was 574 pg/mL and the standard deviation was 721 pg/mL and 56 pg/mL, respectively, for a significance level of 5% and power of 80%, 28 patients would be necessary.

The categorical data were presented as absolute and relative frequency, and continuous data as mean and standard deviation or median and interquartile range, depending on the symmetry of the variables. Chi-square and Fischer's exact were used to compare categorical variables; continuous variables were compared by the Kruskal-Wallis test. For statistical analysis, levels of IFN not detected were counted as zero. The significance level was set at 0.05 and analyses were performed using SPSS v. 17 (SPSS Inc., Chicago, IL, USA).

Written informed consent was obtained from parents or legal caregivers before any study procedure. The study was approved by the Institutional Review Board (number 1.158.826).

4. Results

One hundred five children were initially eligible. Two caregivers did

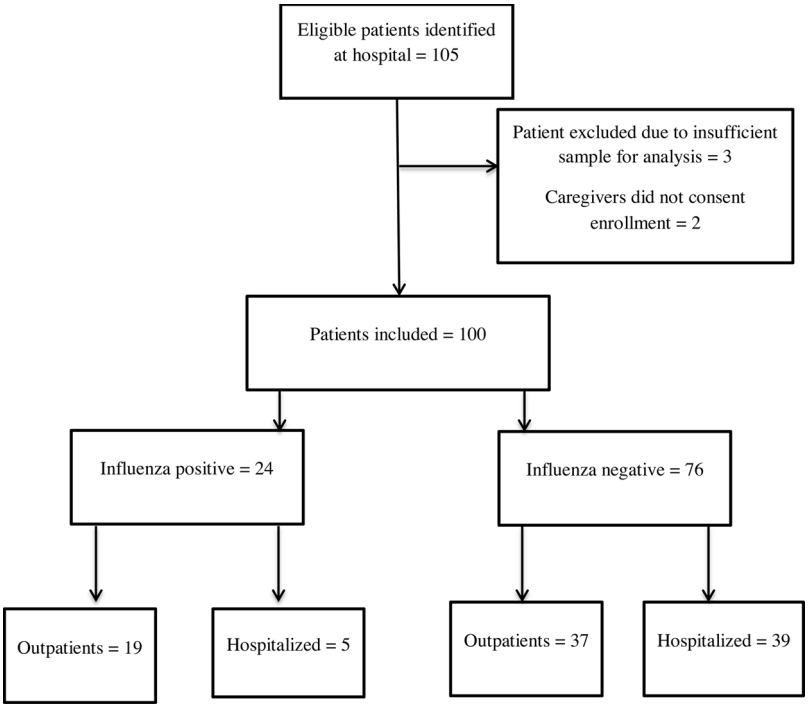

Fig. 1. Flowchart of inclusion and follow-up.

Table 1
Main clinical and demographic characteristics in all four groups compared.

	Patients with influenza		Patients without influenza	
	Outpatients n(%)	Hospitalized n(%)	Outpatients n(%)	Hospitalized n(%)
Age ≤ 6 months*	3 (15.8)	5 (100)	10 (25)	21(53.8)
Gender male	8 (41.2)	5 (100)	25 (62.5)	23 (59)
Comorbidities	5 (26.3)	0 (0)	13 (32.5)	13 (35.1)
Breastfeeding	6 (31.6)	3 (60)	10 (25)	24 (61.5)
Average income < 2 minimum wages**	11 (57.8)	2 (40)	14 (37.8)	21 (53.8)
Race (caucasian)	8 (42.1)	3 (60)	24 (64.8)	25(64.1)

* Age was significantly lower in both hospitalized groups by chi-square ($p < 0.01$). In all other comparisons, the value of "p" was > 0.05.
** Minimum wage in Brazil was 880 Brazilian reais at the time of enrollment.

not accept their children's participation and 3 children were excluded because of the insufficient sample for analysis; thus, 100 children were included. Fifty-three did not require hospitalization. A flowchart of inclusion and follow-up is shown in Fig. 1. Caregivers of all outpatients included were contacted by phone and no further need of hospitalization occurred among outpatients. Main characteristics of the study groups are shown in Table 1. Compared with outpatient children, more hospitalized children were younger than six months (23.2% vs. 59%, respectively, $p < 0.01$). Twenty four patients had influenza infection as confirmed by reverse transcription polymerase chain reaction, 10 with influenza A (H3N2), and 14 with influenza B. All five hospitalized patients with influenza had subtype B. Seventy-six patients (39 needing hospitalization) tested negative for influenza and served as controls. DFA in nasal secretions was performed in 38 hospitalized children and 18 patients from the control group tested positive for RSV.

In the control group, 55 patients were wheezing at enrollment (29 hospitalized). Thorax radiograph was performed in 42 children, 12 of these children (2 outpatients) were classified by hospital radiologist as having lung consolidations and received antibiotics.

As shown in Fig. 2, median levels (pg/mL) of IFN-α2 were significantly higher in outpatients with influenza than in hospitalized patients and were (25–75 interquartile range) 263.2 (58.3–634) and in only one patient it was detectable (90 pg/ml), respectively ($p < 0.001$). The levels of IFN-α2 in the control group were not detectable, regardless of hospitalization. When comparing only influenza B, the differences between the disease severity were similar (data not shown). The levels of IFN-β (pg/mL) were not detectable in all groups.

5. Discussion

To our knowledge, this is one of the first studies assessing nasal levels of IFN-I in children at the beginning of influenza virus infection, comparing more and less severe illness. The differences in the levels of IFN-α2 between these two groups of children reinforce previous experimental findings indicating deficient IFN production might predispose to a more severe influenza infection [22–2528]. Lower levels of IFN-α2 were also found in controls without influenza, regardless of disease severity. It may suggest that IFN-α2 has a more important role in an immune response against influenza virus than against other respiratory viruses, which is in accordance with some reports [29,31–33].

Our study is the first that considers patients mostly with influenza B, which might account for different cytokine levels [34]. The pathogenesis of influenza B innate immune response is less understood, but there are differences [22,35,36]. For example, an early recognition by cell

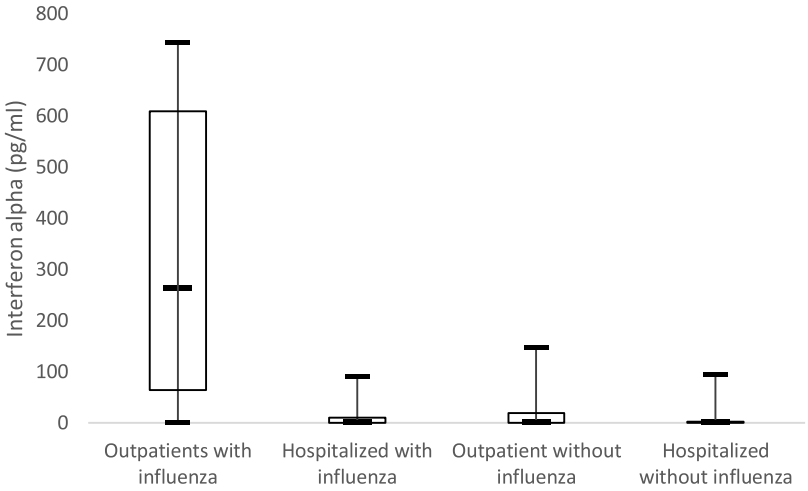

Fig. 2. Comparison among nasal wash levels of IFN-α (pg/ml) in all four groups.
* Levels were significantly higher in outpatients with influenza by Kruskal Wallis ($p < 0.01$).

sensors and faster induction of IFN production has been described after influenza B infection, probably because of less adaptive evolution [37,38].

IFN-β was not detected in all our patients with influenza. In spite of many immune response mechanisms shared with IFN-α, IFN-β is less studied in humans infected by influenza. In pregnant women, a lower expression of IFN-β mRNA was detected in more severe patients, but the levels of IFN-β protein were not measured [39]. In infants with a mild disease, IFN-β was detected only in a small number of patients [29]. Influenza virus can subvert the immune response by several virulence factors that lead to the inhibition of IFN-I production, including IFN-β [40–42]. Moreover, IFN-β mRNA stability is controlled by post-transcriptional mechanisms and the molecules that regulate the integrity and degradation of IFN-β mRNA are poorly characterized [43]. Therefore, the lack of IFN-β detection in our patients might be due to some of these mechanisms, although the exact mechanism remains elusive so far.

Data from experimental studies and growing evidence in humans suggest that impaired production of IFN-I or impaired IFN-induced proteins increase influenza illness severity [23,24]. Interferon-induced transmembrane 3 (IFITM3) has been shown to restrict the entry of enveloped viruses [44]. Three meta-analyses related a specific polymorphism of IFN stimulated gene *IFITM3* (rs12252-C) with an unfavorable course of influenza A infection [45–47]. However, this polymorphism was not associated with the greater severity in children [48]. Compensatory mechanisms as a production of a higher level of antibodies may occur [49]. Moreover, experimental data suggest that the expression of a truncated IFITM3 transcript due to rs12252-C, one of the possible explanations for greater severity, is negligible. Therefore, the mechanisms of association of this polymorphism with severity are not fully understood [50].

In pregnant and elderly patients, the production of IFN-α by peripheral blood mononuclear cells was lower when infected by influenza A virus compared with cells from non-pregnant and non-elderly individuals, respectively [27,28]. In a study assessing nasal levels of IFN-α in infants, mostly outpatients with influenza infection, breastfeeding was independently associated with higher levels of IFN-α, which could be one mechanisms of protection of breast milk [29]. Furthermore, modifications in the main IFN antagonist viral NS1 are extensively described as an attenuating virulence factor and are used for live attenuated vaccine production [51–53]. By contrast, for other respiratory viruses, the studies did not show a significant detection of IFN after infection by these viruses, possibly due to mechanisms of immune response different than those occurring in influenza infection [29,31–33].

Conversely, some studies have related an excessive inflammatory response to the greater severity of influenza A infection in children [22]. In these reports, serum levels of some cytokines, as interleukins–6 and –8 and IFN-γ inducible protein–10, but not IFN-α, were higher in more severe influenza infection [54–56]. Another study assessing cytokine levels in serum and nasal lavage in a population with children and adults found higher levels of nasal IFN-α2 in younger patients and those with higher symptoms scores. Interestingly, serum and nasal levels of many cytokines were poorly correlated [57]. Thus, different fluids compared, as many other factors, might account for controversial findings across studies, as subtype of IFN assessed, sample size, patients' ages, influenza strain, and outcome considered.

This study has limitations. First, because a small number of patients were admitted with influenza, all of these with subtype B, the analysis could be underpowered. Nonetheless, the considerable difference in IFN-α2 levels, the use of a control group, and sub-analysis comparing patients with subtype B give strength to our findings. Second, as hospitalized children were significantly younger, immunological immaturity could account for differences. However, previous evidence suggests that young infants can produce IFN-I in high levels [29,55]. Third, quantification of IFN-α2 in samples obtained through nasal washes might not reflect lower respiratory tract levels, but in a previous study, these levels were found to be similar [58]. Fourth, viral coinfections were not assessed, but, besides the role of IFN-I in influenza may be more prominent than in other respiratory viruses, two meta-analysis showed no increase in disease severity in children with two or more viruses detected [29,59,60]. Fifth, other readout for IFN-I response, as IFN-stimulated genes' activity, were not measured. Lastly, we did not assess if differences found were related to ineffective host response, as a specific polymorphism, or to viral factors, as NS1.

The quality of our data is strengthened due to the prospective cohort design, with a hypothesis made a priori. Furthermore, participants were included at the beginning of illness and followed until recovery, using a control group and findings were in line to the most plausible hypothesis.

Our results give an insight of a protective role of IFN-α2 in the immune response to infection by influenza virus, although studies with a larger sample size are required to confirm our findings. An inverse correlation between nasal levels of IFN-α2 and severity might become a prognostic test, which would be useful since complications can occur in individuals without identified risk factors. Furthermore, this study might guide future intervention studies focused on correcting IFN deficit in children. This has been explored successfully in animal models, but reports of prophylactic use in humans are scarce [61–65]. Further studies are required to validate these findings and assess the role of IFN-α subtypes.

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Competing interests

None declared.

Ethical approval

This study was approved by institutional review board of Hospital São Lucas (number 1.158.826).

CRediT authorship contribution statement

Marcelo Comerlato Scotta: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Supervision, Validation, Visualization, Writing - original draft, Writing - review & editing. **Denise Greff Machado:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing - original draft. **Suelen Goecks Oliveira:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing - original draft. **Angela de Moura:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing - original draft. **Geovana Rhoden Estorgato:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing - original draft. **Ana Paula Duarte de Souza:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Supervision, Validation, Visualization, Writing - original draft, Writing - review & editing. **Barbara Nery Porto:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Supervision, Validation, Visualization, Writing - original draft, Writing - review & editing. **Patrícia Dias de Araújo:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Supervision, Validation, Visualization, Writing - original draft, Writing - review & editing. **Edgar Enrique Sarria:** Conceptualization, Formal analysis, Methodology, Supervision, Validation, Visualization, Writing - original draft. **Paulo Marcio Pitrez:** Conceptualization, Formal analysis, Methodology, Supervision, Validation, Visualization, Writing - original draft. **Marcus Herbert Jones:** Conceptualization, Formal analysis, Methodology, Supervision, Validation, Visualization, Writing - original draft. **Leonardo Araújo Pinto:** Conceptualization, Formal analysis, Methodology, Supervision, Validation, Visualization, Writing - original draft. **Renato Tetelbom Stein:** Conceptualization, Formal analysis, Methodology, Supervision, Validation, Visualization, Writing - original draft. **Fernando P. Polack:** Conceptualization, Formal analysis, Methodology, Supervision, Validation, Visualization, Writing - original draft. **Rita Mattiello:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing - original draft, Writing - review & editing.

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