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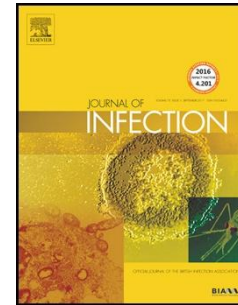
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Author: Kelvin K.W. To, Lu Lu, Carol H.Y. Fong, Alan K.L. Wu, Ka-Yi Mok, Cyril C.Y. Yip, Yi-Hong Ke, Kong-Hung Sze, Susanna K.P. Lau, Ivan F.N. Hung, Kwok-Yung Yuen

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Rhinovirus respiratory tract infection in hospitalized adult patients is associated with T_H2 response irrespective of asthma

Kelvin K. W. To^{1,2,3,4,5}, Lu Lu¹, Carol H. Y. Fong¹, Alan K. L. Wu⁶, Ka-Yi Mok¹, Cyril C. Y. Yip^{1,5}, Yi-Hong Ke¹, Kong-Hung Sze^{1,2,3,4}, Susanna K. P. Lau^{1,2,3,4}, Ivan F. N. Hung^{2,3,4,7}, Kwok-Yung Yuen^{1,2,3,4,5}

¹ Department of Microbiology, Li Ka Shing Faculty of Medicine, The University of Hong Kong, Hong Kong, China.

² Research Centre of Infection and Immunology, The University of Hong Kong, Hong Kong, China.

³ State Key Laboratory of Emerging Infectious Diseases, The University of Hong Kong, Hong Kong, China.

⁴ Carol Yu Centre for Infection, The University of Hong Kong, Hong Kong, China.

⁵ Department of Microbiology, Queen Mary Hospital, Hong Kong, China.

⁶ Department of Pathology, Pamela Youde Nethersole Eastern Hospital, Hong Kong, China.

⁷ Department of Medicine, Li Ka Shing Faculty of Medicine, The University of Hong Kong, Hong Kong, China.

* Correspondence: Kwok-Yung Yuen, Carol Yu Centre for Infection and Division of Infectious Diseases, Department of Microbiology, The University of Hong Kong, Queen Mary Hospital, Pokfulam Road, Pokfulam, Hong Kong Special Administrative Region, China (e-mail: kyyuen@hku.hk).

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Highlights

- Rhinovirus patients had more severe illness than influenza patients.
- A higher eosinophil count was found in patients with rhinovirus infection
- Rhinovirus patients had a blunted cytokine and chemokine response, except IL-5
- IP-10 is a potential biomarker differentiating rhinovirus from influenza patients

SUMMARY

Objectives

We assessed the immunological response of hospitalized adult patients with rhinovirus infection, including critically-ill patients.

Methods

The differential white blood cell (WBC) count and the levels of 29 plasma cytokines/chemokines were compared between 50 adult hospitalized patients with rhinovirus infection and 100 age-matched controls with influenza virus infection.

Results

The demographics and comorbidities were similar between rhinovirus and influenza patients, but severe disease was more common for the rhinovirus cohort. Rhinovirus patients had significantly higher WBC counts than influenza patients, especially for eosinophil ($P=3.1\times 10^{-8}$). The level of the T_H2 cytokine IL-5 was significantly higher among rhinovirus patients, while the levels of 9 other cytokines/chemokines were significantly lower among rhinovirus patients. The levels of CXCL-10 (IP-10), CCL-2 (MCP-1), IFN- α 2, IFN- γ , IL-10, and IL-15 remained significantly lower among rhinovirus patients after correction for multiple comparisons. Notably, CXCL-10 had the highest area-under-the-receiver-operating-characteristic curve (AUC) in differentiating rhinovirus from influenza patients (AUC,0.918). In the patient subgroup without asthma, the difference in the WBC count and cytokine/chemokine levels between rhinovirus and influenza patients remained statistically significant.

Conclusions

Rhinovirus infection was characterized by a prominent T_H2 response, even in patients without asthma. CXCL-10 (IP-10) is a potential biomarker in differentiating rhinovirus from influenza infection.

INTRODUCTION

Rhinovirus is the most frequently detected respiratory pathogen among adult patients with community acquired pneumonia (1). Recent studies indicated that rhinovirus infection has similar or even higher mortality than influenza virus infection (2-4). Rhinovirus has a predilection to cause exacerbation of chronic lung diseases, such as asthma, chronic obstructive pulmonary disease (COPD) and bronchiectasis (5, 6). We have previously shown that critically-ill rhinovirus patients also had severe extrapulmonary complications, including heart failure, diabetic ketoacidosis and seizure (7). Despite being one of the most common causes of severe human infections, there is currently no specific antiviral treatment for rhinovirus infection.

Understanding the immunological response of rhinovirus infection is important in finding effective treatment strategies. Blood eosinophil count was found to be higher among children with rhinovirus infection than those with influenza A(H1N1) or respiratory syncytial virus (RSV) infection (8). Nasal eosinophilia, a hallmark of T_H2 activation, is found in children with rhinovirus infection (9). Studies assessing the levels of cytokines and chemokines *in vitro*, *ex vivo* and *in vivo* have also provided important insights into the pathogenesis of rhinovirus infection (10-12). *In vitro* studies using human airway cell lines and monocytic cells showed that C-X-C motif chemokine 10 (CXCL-10), also known as interferon γ -induced protein 10 (IP-10) was released during rhinovirus infection (13-16). Rhinovirus infection of *ex vivo* human peripheral blood monocytes or bronchoalveolar macrophages also induced the release of CXCL-10 (14). Rhinovirus infection was associated with increased airway levels of CXCL-10 and interleukin 15 (IL-15) in human volunteers (13, 17). Experimental rhinovirus infection in asthmatic subjects induced the release of interferon γ (IFN- γ), CXCL-10, interferon-inducible T-cell-chemoattractant, IL-15 and interleukin 5 (IL-5) in bronchial fluid samples (18). In a study

comparing young adult patients with rhinovirus and influenza virus infection, the serum levels of interferon α/β were detectable only in influenza patients but not in rhinovirus patients (19). The blood level of the CXCL-10 was elevated during rhinovirus-induced asthmatic exacerbation and in patients with COPD (20, 21). Others have examined the blood levels of cytokines and chemokines in patients with rhinovirus and influenza virus infection, with conflicting results. In a study comparing patients of all age groups with mild rhinovirus infection to those with mild or severe influenza virus infection, the levels of most cytokines/chemokines were lower in rhinovirus patients (22). But in another study conducted among adult patients with pneumonia, the levels of most cytokines/chemokines were higher among patients with rhinovirus than those with other respiratory viruses, including influenza virus (23).

Although these studies have provided important data on the immunological response during rhinovirus infection, there are several drawbacks. First, the clinical information regarding the underlying diseases or disease severity was not reported in detail. Age, gender, underlying medical conditions and disease severity can affect the immunological response substantially (24). Second, many studies on rhinovirus infection were performed in children (25), while few studies were conducted for adult patients. The immunological parameters can be significantly different between children and adults. For example, T_H1/T_H2 ratio increases with age and peaks at age 20 to 39 years (26). Third, many studies compared rhinovirus patients with healthy controls. Only few studies have compared the immunological response of rhinovirus patients and those infected with other respiratory viruses (22, 23).

In this study, we sought to elucidate the immunological response of severe rhinovirus infection by comparing the white blood cell (WBC) count and the level of plasma cytokines and chemokines between age-matched patients with rhinovirus infection and those with influenza

virus infection. We included only hospitalized patients, including critically-ill patients. To avoid the effect from confounding factors, we have specifically chosen young- and middle-aged adults, and excluded those with respiratory viral co-infection.

METHODS

Patients

This study included consecutive hospitalized adult patients whose clinical specimens were sent to the clinical microbiology laboratory of Queen Mary Hospital from November 2014 to November 2016, and their respiratory specimens tested positive for influenza A virus, influenza B virus or rhinovirus by either direct immunofluorescence assay (DFA) and/or multiplex PCR. Patients were excluded if they have severe immunocompromising conditions, including transplant recipients, and patients with hematological malignancies, receiving chemotherapy or high dose steroid (prednisolone ≥ 20 mg/day or equivalent). Patients were also excluded if they have respiratory virus co-infections detected in the DFA or multiplex PCR panel, their plasma sample on the 1st day of hospital admission was not available, or their clinical records were not available for review. This study was approved by the Institutional Review Board of the University of Hong Kong/Hospital Authority Hong Kong West and East Clusters (HKU/HA HKW & HKE IRB).

We selected a total of 150 patients infected with rhinovirus, influenza A virus or influenza B virus in a 1:1:1 ratio who were matched for age. For the selected patients, their archived plasma specimens were retrieved from the clinical hematology laboratory and stored at -80°C until testing by cytokine/chemokine assay. Clinical and laboratory information were obtained from the Clinical Management System. Patients were defined as having severe disease

if they required oxygen supplementation, were admitted to the intensive care unit (ICU) or coronary care unit (CCU), or died as we described previously (27).

Respiratory virus detection

DFA was performed using D³[®] Ultra 8[™] DFA Respiratory Virus Screening and Identification Kit (Diagnostic Hybrids, Inc. Quidel, USA) as described previously (28, 29), which included influenza virus (types A and B), parainfluenza virus (types 1, 2, 3), RSV, adenovirus and human metapneumovirus. Multiplex PCR was performed at the Public Health Laboratory Service Branch of Centre for Health Protection, Hong Kong. This multiplex PCR panel included the detection of rhinovirus/enterovirus, influenza virus (types A, B and C), parainfluenza virus (types 1, 2, 3 and 4), RSV and adenovirus (30).

Differentiation between enterovirus and rhinovirus

A patient was considered to have rhinovirus infection if the real-time reverse transcription-quantitative polymerase chain reaction (RT-qPCR) was positive for rhinovirus but negative for enterovirus, using specific primers and probes as described previously (31) (Table 1). The RT-qPCR for rhinovirus can detect rhinovirus species A, B and C. RT-qPCR was carried out using AgPath-ID One-Step RT-PCR reagents (Applied Biosystems, USA) (29, 31). The reagent mixture (25 µl) contained 1X RT-PCR Buffer, 1X RT-PCR Enzyme Mix, 0.4 µM forward and reverse primers, 0.12 µM probe, 5 µl of total nucleic acid as template and nuclease-free water. The thermal cycling conditions were 10 min at 45 °C for reverse transcription and then 10 min at 95 °C for reverse transcription inactivation/initial denaturation, followed by 50

cycles of 15 s at 95 °C, and 45 s at 55 °C. All reactions were performed using LightCycler® 96 Real-time PCR System (Roche, Switzerland).

Cytokine/chemokine assay

The plasma levels of 29 cytokines/chemokines were measured using MILLIPLEX® MAP Human Cytokine/Chemokine Magnetic Bead Panel (Merck Millipore, USA) (HCYTMAg-60K-PX29) according to manufacturer's instructions as we described previously (11, 32, 33). This panel includes the following cytokines and chemokines: C-C Motif Chemokine Ligand (CCL)-2 (CCL-2) (also known as monocyte chemoattractant protein-1 [MCP-1]), CCL-3 (macrophage inflammatory protein [MIP]-1 α), CCL-4 (MIP-1 β), CCL-11 (eotaxin-1), C-X-C Motif Chemokine Ligand (CXCL)-8 (CXCL-8) (also known as IL-8), CXCL-10 (IP-10), epidermal growth factor (EGF), granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), interferon α 2 (IFN- α 2), IFN- γ , interleukin (IL)-1 α , IL-1 β , IL-1RA, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-10, IL-12p40, IL-12p70, IL-13, IL-15, IL-17A, tumor necrosis factor (TNF)- α , TNF- β , and vascular endothelial growth factor (VEGF) (Supplementary Table S1). The plates were read using the Luminex® 200™ instrument. The data were analyzed using MILLIPLEX Analyst v.5.1.

Statistical analysis

IBM SPSS Statistics 23.0.0.0 was used for statistical computations unless otherwise specified. Mann Whitney *U* test and Chi Square test were used in the comparison of continuous and categorical variables, respectively. Kruskal Wallis test was used for categorical variables when comparing 3 groups. The overall cytokine and chemokine profile was compared between

rhinovirus and influenza patients by partial-least squares discriminant analysis (PLS-DA) using SIMCA-P13.0 software (Umetrics) as we described previously (34). Receiver operating characteristic (ROC) curve analysis was performed using GraphPad Prism 6 for Windows (version 6.07). If the level of cytokine/chemokine was below the limit of detection, half of the lower limit of detection was used for statistical analysis. A *P* value of <0.05 was considered to be statistically significant. Bonferroni correction was used to correct for multiple comparisons for the cytokine and chemokine levels.

RESULTS

Patient characteristics

A total of 150 hospitalized adult patients were included in this study, comprising 50 patients with rhinovirus infection, and 100 patients with influenza virus infection (50 patients with influenza A virus and 50 patients with influenza B virus infection). Among the patients with influenza A virus infection, 43 patients had H3N2 subtype and 7 patients had H1N1 subtype. The male-to-female ratio was 1.24:1. The median age was 50 years, with an interquartile range from 37 to 58 years. Forty five patients (30%) did not have any known chronic medical illness. There were no statistically significant differences in the age, gender or comorbidities among rhinovirus, influenza A or influenza B patients, or between rhinovirus and influenza patients (Table 2).

Twenty eight patients (18.7%) had severe disease, including 27 patients (18%) who required oxygen supplementation, 6 patients (4%) who required positive pressure ventilation (PPV), 7 patients (4.7%) who were admitted to ICU or CCU, and 2 patients (1.3%) who died (Table 2). Ten patients (6.7%) had co-infections with pulmonary bacterial/fungal pathogens or non-respiratory viruses. A significantly higher proportion of rhinovirus patients had exacerbation

of chronic lung disease ($P = 0.012$) and severe disease ($P = 0.002$) than those with influenza A or influenza B virus infection. There were no significant differences in the proportion of patients with pneumonia. There were no significant differences in the clinical syndromes or clinical severity between influenza A or influenza B patients.

Comparison of WBC count between rhinovirus and influenza patients

The total WBC, neutrophil, lymphocyte, and eosinophil counts were significantly higher among rhinovirus patients than influenza A or influenza B patients (Table 3). The difference between rhinovirus patients and influenza virus patients was most statistically significant for eosinophil count ($P = 3.1 \times 10^{-8}$). Since asthma patients may have a higher eosinophil count, we have analyzed the subgroup of patients without asthma. Among patients without asthma, rhinovirus patients still had a significantly higher eosinophil count than influenza patients (median eosinophil count [interquartile range]: $0.105 \times 10^9/L$ [$0.03-0.238 \times 10^9/L$] vs $0.01 \times 10^9/L$ [$0.03-0.23 \times 10^9/L$]; $P = 9.8 \times 10^{-7}$). Neutrophilia and eosinophilia were significantly more common among rhinovirus patients than influenza patients, while lymphopenia was less common for rhinovirus patients. Notably, eosinophilia $\geq 0.5 \times 10^9/L$ was uncommon even for rhinovirus patients (8.3%). The difference in the WBC counts between rhinovirus and influenza patients remained statistically significant even after excluding patients with co-infections. There were no significant differences in the total WBC or differential WBC counts between influenza A or influenza B patients.

Comparison of cytokine/chemokine profile between rhinovirus and influenza patients

Partial-least squares discriminant analysis (PLS-DA) was used in comparing the overall cytokine/chemokine profile between the different patient cohorts (Figure 1). In PLS-DA, each patient is represented by a single dot in a 3-dimensional space in such a manner that the closer dots/patients are, the more they share similar cytokine/chemokine profiles. The levels of all cytokines/chemokines measured were included in the analysis. Rhinovirus patients formed a separate cluster from influenza patients in PLS-DA plots (Figure 1A and 1B), while there was no clear separation between influenza A and influenza B patients (Figure 1C). Therefore, we only performed the comparison between rhinovirus patients and all influenza patients for cytokine and chemokines in subsequent analysis.

Rhinovirus patients had a significantly higher level of the T_H2 cytokine IL-5 than influenza patients ($P = 0.0112$) (Figure 2). However, rhinovirus patients had significantly lower levels of hematopoietic growth factor (GM-CSF), chemokines (CXCL-8, CXCL-10 and CCL-2), interferons (IFN- $\alpha 2$ and IFN- γ), pro-inflammatory cytokine (TNF- α), anti-inflammatory cytokine (IL-10) and T_H1 -associated cytokine (IL-15). For patients without asthma, the level of IL-5 was still significantly higher for rhinovirus patients than influenza patients ($P = 0.029$). After Bonferroni correction, only CXCL-10, CCL-2, IFN- $\alpha 2$, IFN- γ , IL-10, IL-15 remained statistically significant. Since co-infections may affect the cytokine and chemokine levels, we re-analyzed the data after excluding the patients with known co-infections. CXCL-10, CCL-2, IFN- $\alpha 2$, IFN- γ , IL-10, IL-15 remained significantly different when patients with co-infections were excluded (Figure 3).

For subgroup analysis of patients with severe disease without co-infections, the levels of IL-10, CXCL-8, CXCL-10, IFN- $\alpha 2$, CCL-2 and TNF- α remained significantly lower in patients

with rhinovirus infection than those with influenza virus infection, while the level of IL-5 was higher among rhinovirus patients than those of influenza patients (Supplementary Figure S1).

ROC analysis was performed for the cytokines or chemokines with significant difference between rhinovirus and influenza patients. CXCL-10 has the highest area under curve (AUC) of 0.918 (Table 4 and Figure 4). When a cutoff of 1745 pg/ml was used, the sensitivity and specificity was 89% and 88%, respectively.

Subgroup analysis for rhinovirus patients

We further analyzed the immunological profile of rhinovirus patients without any identifiable co-infections. There was no significant difference in eosinophil count and the levels of the tested cytokines/chemokines between patients with or without asthma or other chronic lung disease, or between patients with or without exacerbation of underlying lung disease.

DISCUSSION

Both rhinovirus and influenza virus predominantly cause respiratory tract infection, and often accompanied by systemic complications (35, 36). However, the pathogenesis of rhinovirus and influenza virus infection is markedly different. While influenza virus causes cytopathic effect in airway epithelial cells, rhinovirus usually leads to epithelial barrier disruption with little cytopathological changes (37). This study compared age-matched hospitalized adult patients with rhinovirus infection and those with influenza virus infection who have similar demographics and underlying medical conditions. Consistent with our previous findings (2), rhinovirus patients had significantly higher morbidity and mortality than influenza patients in the current cohort. Rhinovirus patients had significantly higher WBC count than influenza patients,

including neutrophil, lymphocyte and eosinophil counts. The difference in eosinophil count was most significant, with a P value of 10^{-8} . Despite having more severe disease, rhinovirus infection was characterized by a blunted cytokine and chemokine response when compared with influenza infection. The levels of hematopoietic growth factor (GM-CSF), chemokines (CXCL-10, CXCL-8 and CCL-2), type I and type II interferons (IFN- α 2 and IFN- γ), pro-inflammatory cytokines (TNF- α and IL-15), and anti-inflammatory cytokine (IL-10) were lower among rhinovirus patients than those of influenza patients. The only exception was the level of the T_H2 cytokine IL-5, which was significantly higher among rhinovirus patients than influenza patients. Notably, the difference in eosinophil count and the level of IL-5 remained significantly different even for patients without asthma. Our findings suggest that adult patients with rhinovirus infection are characterized by a T_H2 type response with limited systemic inflammatory response, even for patients without asthma.

The current study differs from previous studies in several aspects. First, we examined hospitalized adult patients with natural infection, which included critically-ill patients requiring PPV or ICU admission. Studying patients with severe disease is important because effective treatment has the highest impact in this group of patients. Second, we have selected age-matched subjects since age may affect cytokine and chemokine response, and elderly is more likely to have more comorbidities which may affect the immunological response (24). Since there was no significant difference in age, gender and comorbidities between rhinovirus and influenza patients, the difference in blood WBC count and cytokines/chemokines are unlikely to be related to these potential confounders. Third, we performed subgroup analysis for patients without known bacterial or viral co-infections. Therefore, the difference in blood eosinophil count and cytokine/chemokine profile is unlikely to be related to the other pathogens.

Although most patients had eosinophil counts within the normal range, rhinovirus patients had a significantly higher eosinophil count than influenza patients, with a P value of 10^{-8} . This is consistent with the findings in a pediatric study, in which the blood eosinophil count was higher among infants with rhinovirus infection than those with influenza virus or RSV infection (8). The higher blood eosinophil count corroborates with the higher level of IL-5 among rhinovirus patients when compared with influenza patients. Out of the 29 cytokines tested, IL-5 was the only cytokine/chemokine with a significantly higher plasma level in rhinovirus patients than in influenza patients, although the P value was slightly above the cutoff of 0.05 after Bonferroni correction for multiple comparison. IL-5 is a type 2 cytokine which contributes to the proliferation, maturation, migration and survival of eosinophils (38). IL-5 is important in the pathogenesis of asthmatic exacerbations, and IL-5 inhibitor has been evaluated in clinical trials. In two phase 3 clinical trials, subcutaneous benralizumab, an IL-5 inhibitor, has been shown to reduce the frequency of asthmatic exacerbations in patients with severe asthma (39, 40).

In our study, the difference in eosinophil count and IL-5 remained significantly different between rhinovirus and influenza patients without asthma, suggesting that rhinovirus may directly trigger a systemic T_H2 response even in non-asthmatic patients. Indeed, Singh *et al* has shown that rhinovirus protease 2A could directly trigger T_H2 response in mice (6). The production of IL-5 is also regulated by type I interferon that is secreted by innate immune cells such as dendritic cells (41). This is compatible with our observation that the level of IFN- $\alpha 2$ was significantly lower in rhinovirus patients than influenza patients.

The relatively blunted cytokine/chemokine response of rhinovirus infection, when compared with influenza infection, concurs with findings from previous gene expression studies.

Zhai *et al* showed in a blood microarray gene expression analysis that patients naturally infected with rhinovirus had a lower level of gene enrichment in the interferon signaling and TNF production pathway than those with influenza virus infection (42). Experimental infection in cystic fibrosis patients has shown that fewer antiviral genes were expressed during rhinovirus infection than influenza infection (43). The blunted cytokine response in rhinovirus patients may be related to the increased expression of suppressor of cytokine signaling 1 (SOCS1) gene, which regulates cytokine signaling. Zaas AK *et al* showed that SOCS1 was uniquely expressed in volunteers experimentally infected with rhinovirus but not expressed in volunteers experimentally infected with influenza virus or RSV (44).

In this study, the levels of the chemokines CXCL-10 (IP-10), CXCL-8 (IL-8) and CCL-2 (MCP-1) were significantly lower among rhinovirus patients than influenza patients. These chemokines attract neutrophils, macrophages and other immune cells. High levels of these chemokines in blood or airway have been associated with severe respiratory virus infection (11, 45, 46). The difference in the plasma level of CXCL-10 between rhinovirus and influenza patients was particularly striking, with a P value of 8.2×10^{-17} and an AUC of 0.918 in the ROC curve analysis, making it a potential biomarker differentiating rhinovirus from influenza virus infection. Previous studies have demonstrated that serum CXCL-10 was a marker of rhinovirus infection in patients with exacerbations of asthma or COPD (20, 21). However, these studies did not compare the level of CXCL-10 between patients with rhinovirus infection and those with influenza virus infection.

Rhinovirus has been intensively studied in patients with asthma. When comparing between asthmatic and non-asthmatic individuals, experimental rhinovirus infection in asthmatic subjects induced higher bronchial fluid levels of IFN- γ , CXCL-10, IL-15 and IL-5 than in non-

asthmatic subjects (18). However, in our study, there were no significant differences in these cytokines or chemokines in rhinovirus patients with or without asthma. Therefore, rhinovirus may have exaggerated cytokine and chemokine response in the airway of asthmatic individuals, but the systemic response of asthmatic individuals is similar to those of non-asthmatic individuals.

The plasma levels of type I interferon (IFN- α 2) and type II interferon (IFN- γ) were lower among rhinovirus patients than influenza patients. In particular, IFN- α 2 is the 2nd most discriminatory cytokine, with a *P* value of 5×10^{-6} . Our result concurs with those in the study by Makela *et al*, in which type I interferon was not detected in the serum of adult patients with rhinovirus infection (19). The lack of systemic type I interferon response in rhinovirus patients may contribute to the severity of disease. Agrati *et al* has previously shown that patients with severe influenza infection had lower levels of IFN- α than those with mild disease (47).

This study only included young adult patients. The aim is to minimize the number of confounding factors, such as older age and comorbidities. The immune response of rhinovirus in hospitalized elderly patients should be further elucidated in future studies.

Severe influenza virus infection is accompanied by a dysregulated cytokine and chemokine response (11). Immunomodulators have been shown to improve survival in animal or human studies for influenza virus (48, 49). Unlike influenza, the cytokine and chemokine profile of adult patients with severe rhinovirus infection was not known. In our subgroup analysis, severe rhinovirus patients still had a blunted cytokine response when compared with influenza patients. Therefore, systemic immunomodulators which affect the pro-inflammatory or anti-inflammatory cytokine response may not be the optimal strategy in treatment rhinovirus infection. In contrast, our study showed that hospitalized patients with rhinovirus infection have

a T_H2 -biased response even in patients without asthma. Treatment of rhinovirus infection by targeting the T_H2 response should be further investigated in the future.

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FIGURE LEGEND

Figure 1. 3D score plots of partial-least squares discriminant analysis of the cytokine and chemokine profiles of patients with rhinovirus, influenza A virus and influenza B virus infection. -t[1]-t[3] represent the first three principal components calculated using PLS-DA.

A) Comparison among patients with rhinovirus, influenza A virus and influenza B virus infection;

B) Comparison between patients with rhinovirus and all influenza patients;

C) Comparison between patients with influenza A virus and influenza B virus infection.

Abbreviations: FluA, patients with influenza A virus infection; FluB, patients with influenza B virus infection; FluA/B, patients with influenza A or influenza B virus infection; RV, Rhinovirus.

Figure 2. Comparison of the levels of cytokines and chemokines between all rhinovirus and influenza patients. Mann Whitney U test was used in the comparison of the levels of cytokines/chemokines.

Abbreviations: FluA/B, patients with influenza A or influenza B virus infection; RV, Rhinovirus.

Figure 3. Comparison of the levels of cytokines and chemokines between rhinovirus and influenza patients without co-infections. Mann Whitney U test was used in the comparison of the levels of cytokines/chemokines.

569 Abbreviations: FluA/B, patients with influenza A or influenza B virus infection; RV, Rhinovirus.

570

571 **Figure 4.** Receiver operating characteristic (ROC) curve analysis of CXCL-10 (IP-10)

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Table 1. Primers and probes used in the present study

Virus	Primer/probe sequence (5' - 3')	
Rhinovirus	Forward 1	AGCCYGCGTGGCKGCC
	Forward 2	AGCCYGCGTGGTGCCC
	Reverse	GAAACACGGACACCCAAAGTAGT
	Probe	HEX-TCCGGCCCCTGAATGYGGCTAA-BHQ1
Enterovirus	Forward	GCCCCTGAATGCGGCTAAT
	Reverse	ATTGTCACCATAAGCAGYCA
	Probe	FAM-CGGACACCCAAAGTAGTCGGTTCCG-BHQ1

Table 2. Characteristics of patients included in this study

	FluA (n=50)	FluB (n=50)	FluA/B^a (n=100)	RV (n=50)	P (RV vs FluA vs FluB)	P (RV vs FluA/B)
Demographics						
Median age in years (range)	52 (24-64)	50 (19-64)	51 (19-64)	49 (19-65)	N.S	N.S
Female gender	28 (56)	29 (58)	57 (57)	26 (52)	N.S	N.S
Comorbidity						
No chronic medical illness	16 (32)	14 (28)	30 (30)	15 (30)	N.S	N.S
Hypertension	12 (24)	9 (18)	21 (21)	9 (18)	N.S	N.S
Chronic cardiac disease	5 (10)	8 (16)	13 (13)	5 (10)	N.S	N.S
Chronic pulmonary disease	8 (16)	7 (14)	15 (15)	12 (24)	N.S	N.S
Asthma	5 (10)	7 (14)	12 (12)	11 (22)	N.S	N.S
Chronic renal disease	4 (8)	2 (4)	6 (6)	1 (2)	N.S	N.S
Chronic hepatic disease	1 (2)	2 (4)	3 (3)	1 (2)	N.S	N.S
Diabetes mellitus	9 (18)	11 (22)	20 (20)	12 (24)	N.S	N.S
Thyroid disease	5 (10)	1 (2)	6 (6)	4 (8)	N.S	N.S
Neurological conditions	3 (6)	2 (4)	5 (5)	3 (6)	N.S	N.S
Malignancy	1 (2)	3 (6)	4 (4)	4 (8)	N.S	N.S
Autoimmune disease	0 (0)	2 (4)	2 (2)	2 (2)	N.S	N.S
Clinical syndrome						
Pneumonia	6 (12)	1 (2)	7 (7)	6 (12)	N.S	N.S
Exacerbation of chronic lung disease	3 (6)	4 (8)	7 (7)	12 (24)	0.012	0.007
Extrapulmonary manifestations	6 (12)	4 (8)	10 (10) ^b	14 (28) ^c	0.016	0.008
Co-infection with bacterial/fungal pathogens or non- respiratory viruses	3 (6)	1 (2)	4 (4) ^d	6 (12) ^e	N.S	N.S
Clinical severity						
Severe disease ^f	7 (14)	4 (8)	11 (11)	17 (34)	0.002	0.001
Require oxygen supplementation	6 (12)	4 (8)	10 (10)	17 (34)	0.001	0.001
Require PPV	2 (4)	1 (2)	3 (3)	3 (6)	N.S	N.S
ICU or CCU admission	4 (8)	1 (2)	5 (5)	2 (4)	N.S	N.S.
Death	0 (0)	0 (0)	0 (0)	2 (4)	N.S	N.S.

Data are expressed as number (%) unless otherwise stated.

Abbreviations: CCU, coronary care unit; FluA, influenza A virus; FluA/B, influenza A or influenza B virus; FluB, influenza B virus; ICU, intensive care unit; N.S, not significant; PPV, positive pressure ventilation; RV, rhinovirus.

^aThere was no statistically significant differences in demographics, comorbidity, clinical syndrome and clinical severity between influenza A and influenza B patients.

^bExtrapulmonary manifestations for influenza patients include heart failure/acute pulmonary edema, pericardial effusion with tamponade, hypoglycemia, diabetic ketoacidosis, urinary tract infection, generalized tonic clonic seizure, vasovagal attack, acute hepatitis, esophagitis

^cExtrapulmonary manifestations for rhinovirus patients include fluid overload, gastroenteritis, erythema nodosum, hyperglycemia, atrial fibrillation, SVT, generalized tonic clonic seizure, syncope, hypertensive crisis, immune thrombocytopenic purpura, infectious mononucleosis

^dInvasive aspergillosis (1 patient); *Pseudomonas aeruginosa* from sputum (1 patient); *Haemophilus influenzae* from sputum (1 patient), *Staphylococcus aureus* from sputum (1 patient)

^e*Haemophilus influenzae* from sputum (1 patient), *Klebsiella* species and *Staphylococcus aureus* from sputum (1 patient); *Streptococcus pneumoniae* from sputum (1 patient); *Acinetobacter baumannii* from blood and tracheal aspirate (1 patient); rotavirus gastroenteritis (1 patient); cytomegalovirus infectious mononucleosis-like syndrome (1 patient)

^fRequired oxygen supplementation, admitted to intensive care unit, or died

Table 3. Comparison of the white blood cell counts between rhinovirus and influenza patients

WBC	All patients						Exclude co-infections						AUC	
	FluA ^a	FluB	FluA/B	RV ^a	P ^c (RV vs FluA vs FluB)	P (RV vs FluA/B)	FluA ^a	FluB	FluA/ FluB	RV ^a	P ^c (RV vs FluA vs FluB)	P (RV vs FluA/B)	All patients	Exclude co- infection
Total, × 10 ⁹ /L ^a	6.94 (2.39- 15.31)	5.26 (3.94- 7.75)	6.12 (4.29- 8.16)	9.49 (6.87- 11.69)	1.0E-6	4.7E-7	6.92 (4.81- 9.01)	5.24 (3.93- 7.48)	6.04 (4.27- 8.15)	9.62 (7.03- 11.82)	2.7E-7	1.4E-7	0.746	0.778
Neu, × 10 ⁹ /L ^a	5.53 (3.78- 7.02)	3.74 (2.65- 5.74)	4.53 (2.94- 6.31)	7.16 (4.47- 9.68)	2.1E-5	4.4E-5	5.56 (3.64- 7.03)	3.65 (2.64- 5.56)	4.35 (2.91- 6.27)	7.51 (4.62- 10.07)	0.004	5.0E-6	0.710	0.746
Neu >7.5× 10 ⁹ /L ^{a,b}	7 (15.2)	8 (16.7)	15 (16.0)	23 (47.9)	0.0003	0.0001	7 (16.3)	7 (14.9)	14 (15.6)	21 (50)	0.0002	8.7E-5	N.A	N.A
Lym, × 10 ⁹ /L ^a	0.84 (0.51- 1.10)	0.97 (0.74- 1.16)	0.90 (0.64- 1.12)	1.24 (0.84- 1.76)	0.004	0.002	0.79 (0.50- 1.09)	0.97 (0.74- 1.15)	0.89 (0.62- 1.12)	1.27 (0.86- 1.78)	0.001	0.0004	0.662	0.693
Lym <1.1× 10 ⁹ /L ^{a,b}	34 (73.9)	34 (70.8)	68 (72.3)	20 (41.7)	0.002	0.001	32 (74.4)	33 (70.2)	65 (72.2)	16 (38.1)	0.001	0.0003	N.A	N.A
Eos, × 10 ⁹ /L ^a	0.01 (0- 0.03)	0.02 (0- 0.05)	0.01 (0-0.04)	0.11 (0.03- 0.23)	1.9E-7	3.1E-8	0.01 (0- 0.03)	0.02 (0- 0.05)	0.01 (0- 0.04)	0.125 (0.03- 0.27)	2.8E-7	4.7E-8	0.782	0.793
Eos ≥ 0.5 × 10 ⁹ /L ^{a,b}	1 (2.2)	0 (0)	1 (1.1)	4 (8.3)	N.S	0.045	1 (2.3)	0 (0)	1 (1.1)	4 (9.5)	N.S	0.035	N.A	N.A

Data expressed as median (interquartile range) unless otherwise stated.

Abbreviations: AUC, area under curve; Eos, eosinophil; FluA, influenza A virus; FluA/B, influenza A or influenza B virus; FluB, influenza B virus; Lym, lymphocyte; N.A, not applicable; Neu, neutrophil; RV, rhinovirus; WBC, White blood cell

^a Only included patients with differential white blood cell count available for the first blood sample collected upon admission (All patients: influenza group, n=94; rhinovirus group, n=48; Exclude co-infections: influenza group, n=90; rhinovirus group, n=44)

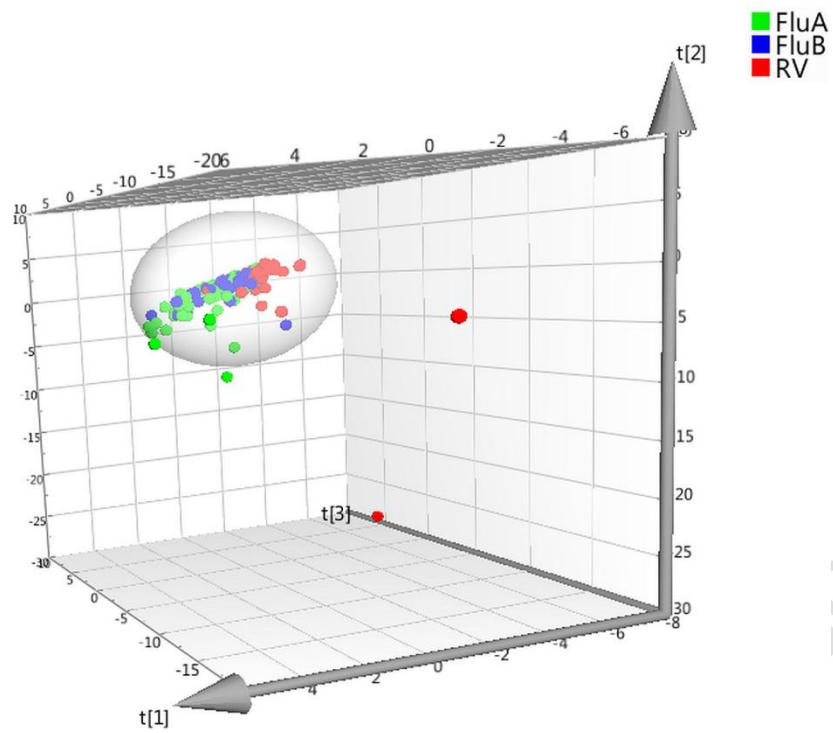
^b Data expressed as no. (%)

^c No statistically significant difference between influenza A and influenza B except for the neutrophil count. All patients; $P = 0.016$; Exclude co-infections, $P = 0.012$

Table 4. Comparison of the levels of cytokines and chemokines between rhinovirus and influenza patients

Cytokines/ chemokines	AUC	
	All patients	Exclude co-infection
Chemokine		
CXCL-10 (IP-10)	0.918	0.937
CCL-2 (MCP-1)	0.677	0.689
CXCL-8 (IL-8)	0.617	0.654
Interferons		
IFN- α 2	0.712	0.740
IFN- γ	0.662	0.691
Pro-inflammatory cytokine		
TNF- α	0.611	0.641
Anti-inflammatory cytokine		
IL-10	0.689	0.706
T_H1 cytokine		
IL-15	0.643	0.661
T_H2 cytokine		
IL-5	0.580	0.595
Hematopoietic growth factor		
GM-CSF	0.617	0.656

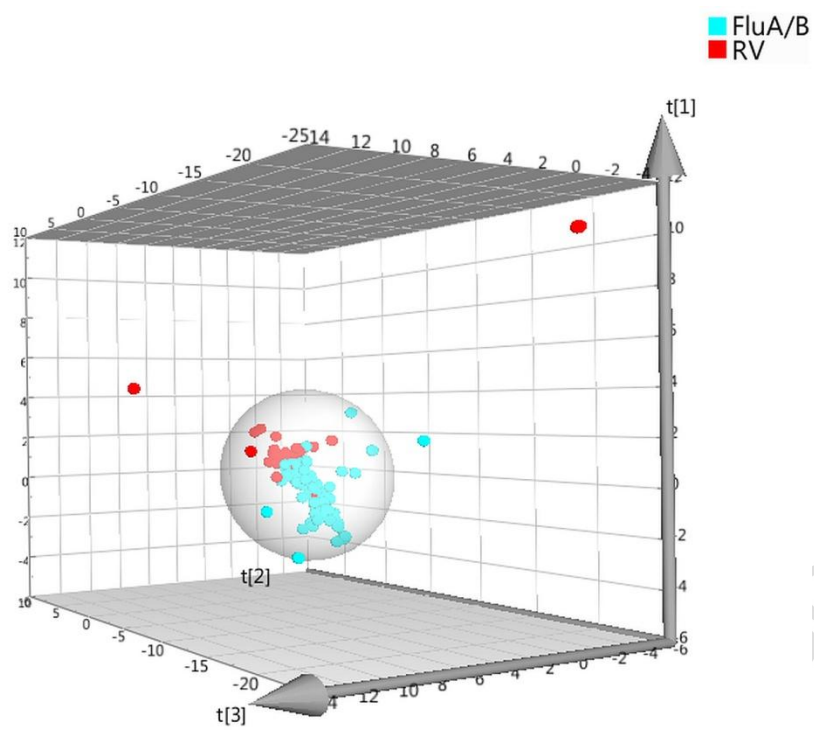
Abbreviations: AUC, area-under-the-receiver-operating-characteristic curve; CCL2, C-C Motif Chemokine Ligand-2; CXCL-8, C-X-C Motif Chemokine Ligand 8; CXCL-10, C-X-C motif chemokine 10; GM-CSF, granulocyte macrophage colony-stimulating factor; IL-10, interleukin 10; IL-15, interleukin 15; IL-5, interleukin 5, IL-8, interleukin 8; IP-10, IFN- γ -inducible protein 10; IFN- α 2, interferon- α 2; IFN- γ , interferon- γ ; MCP-1, monocyte chemoattractant protein-1; TNF- α , tumor necrosis factor- α



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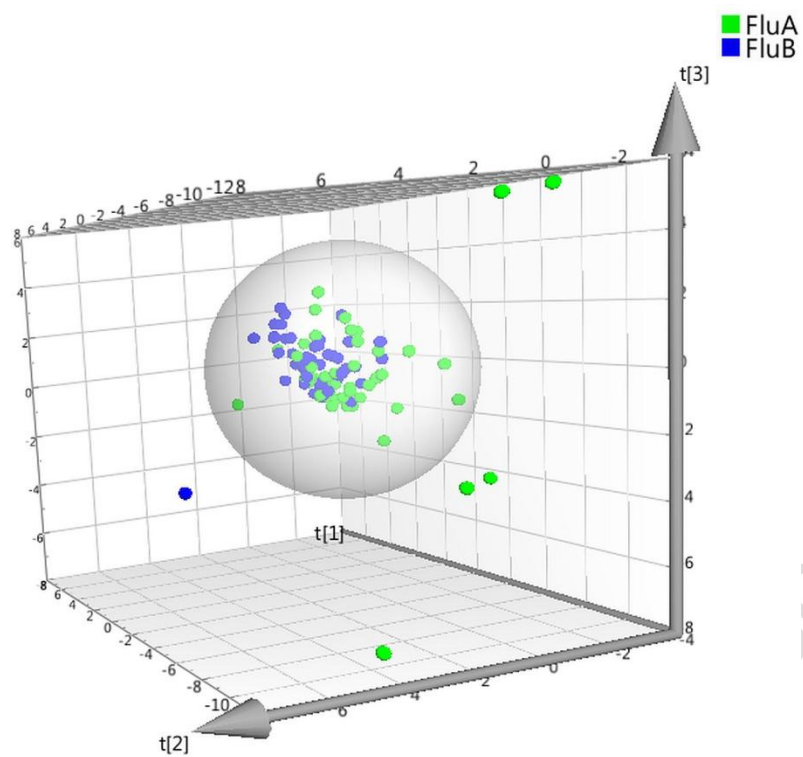
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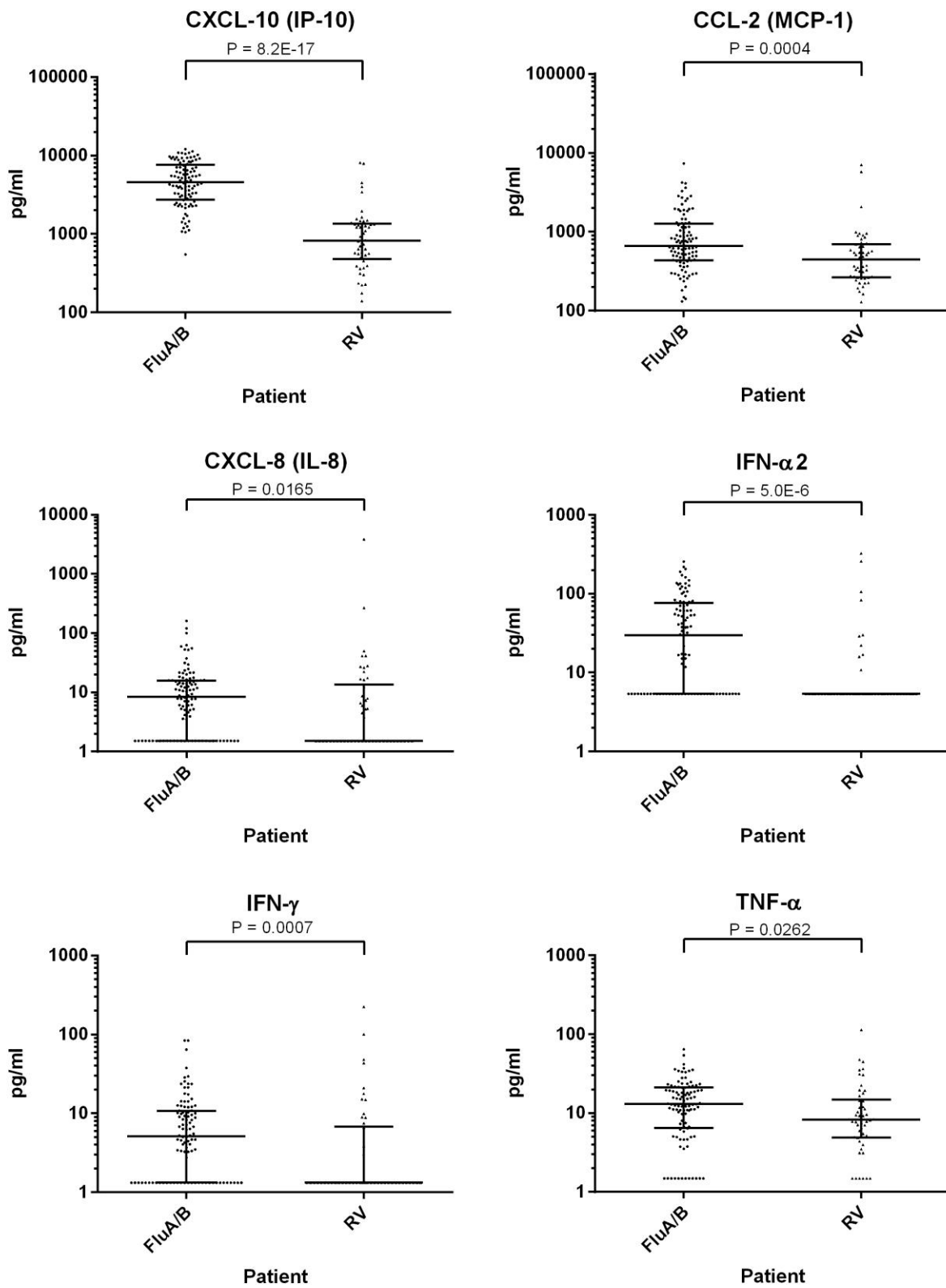
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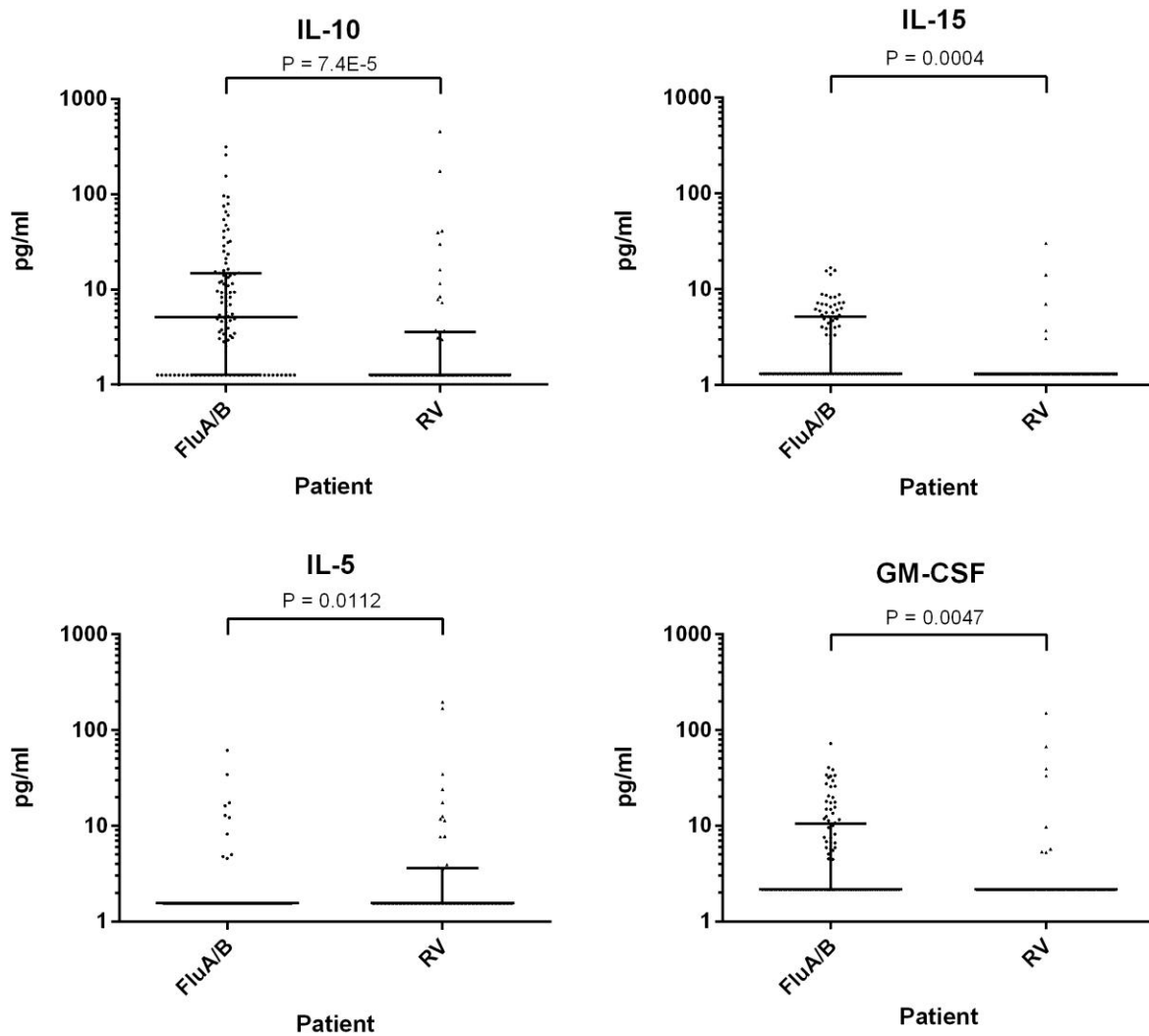
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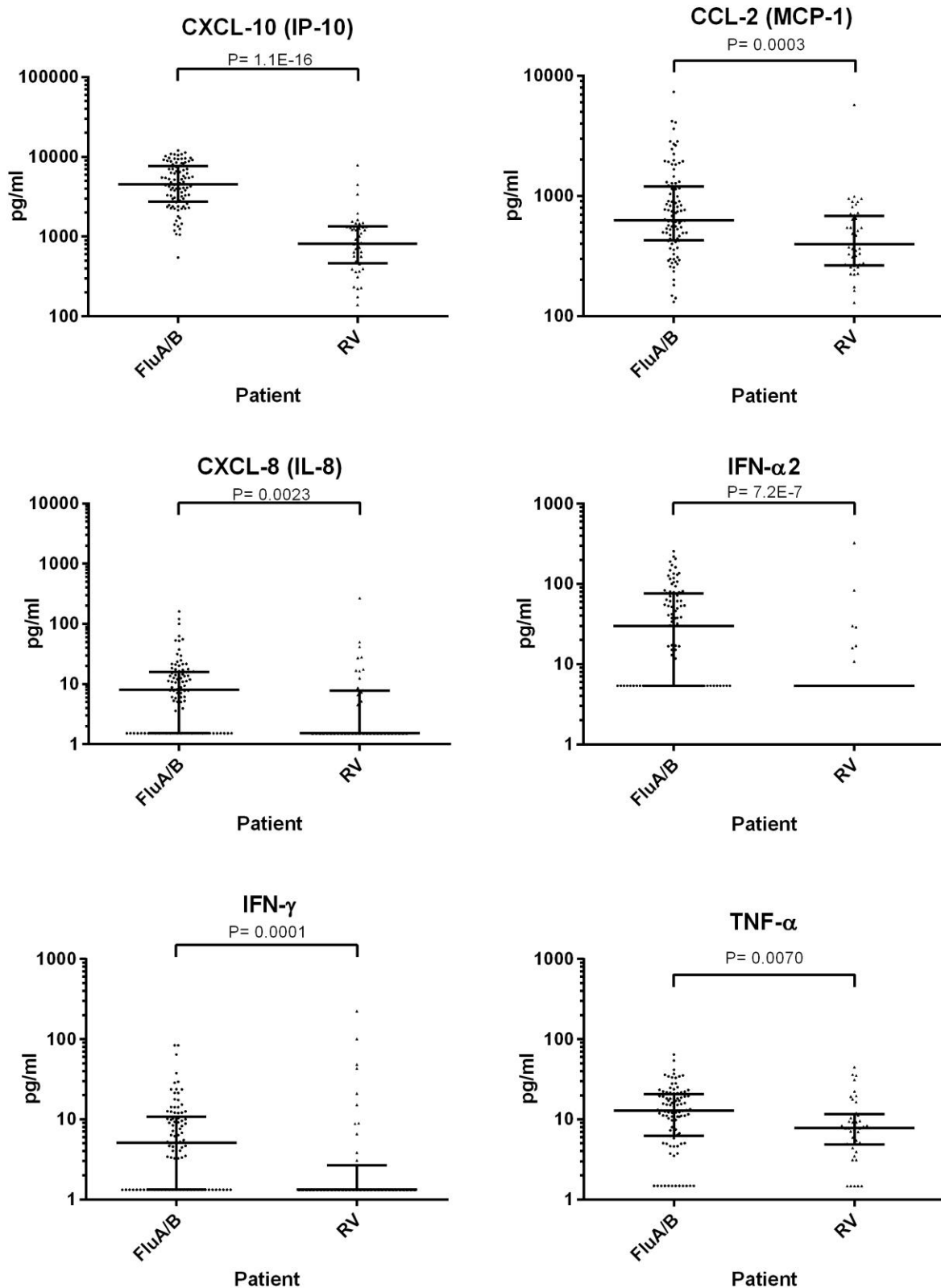
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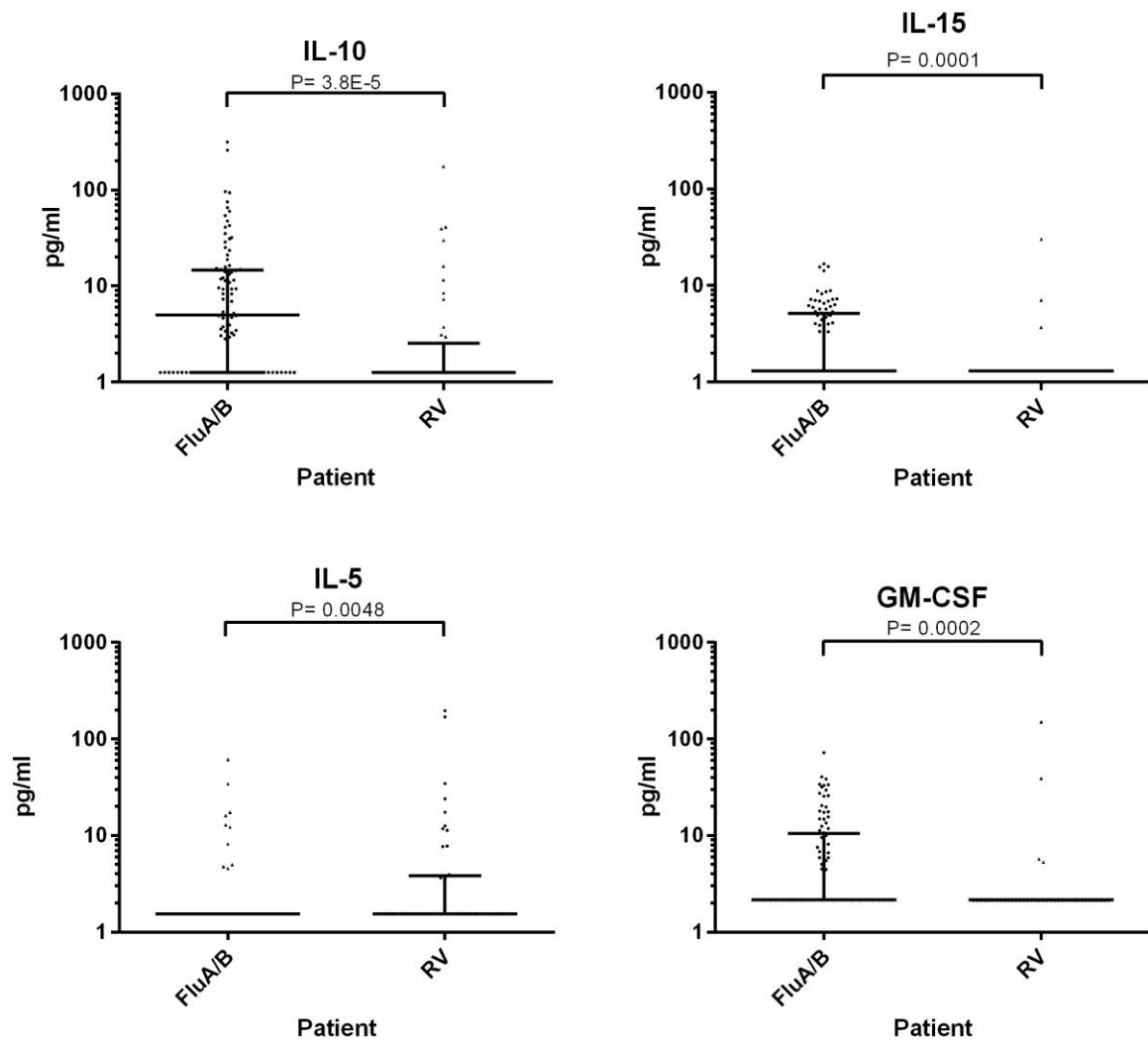
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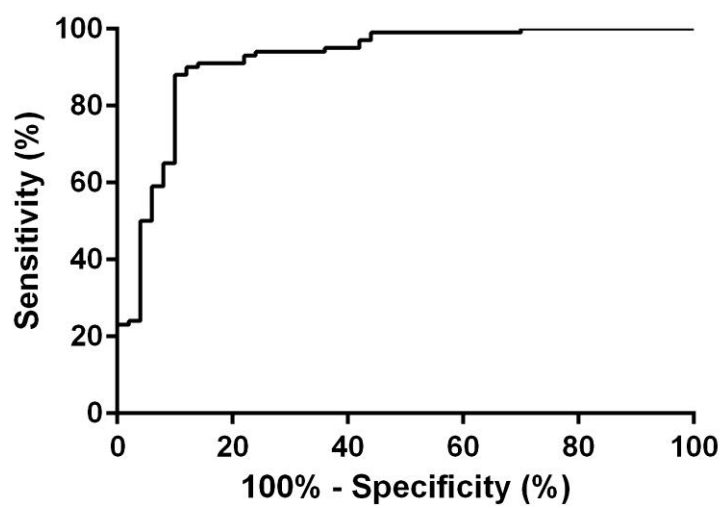
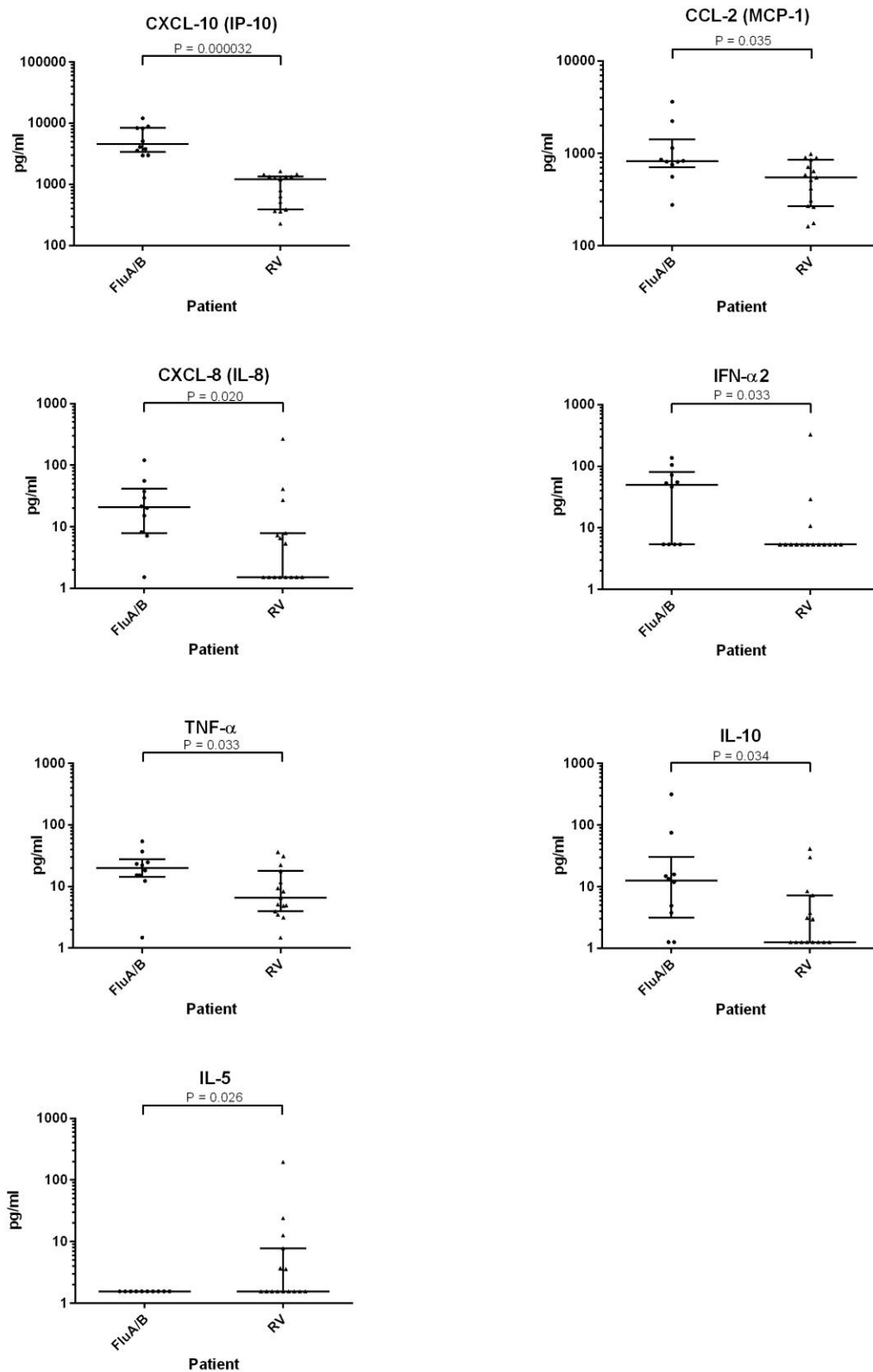


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