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Short Communication

Low peripheral blood *chemokine (C-C motif) ligand 5* and *tumor necrosis factor α* gene expression is associated with unfavorable progression of respiratory syncytial virus bronchiolitis in infants

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ABSTRACT

Objectives: We aimed to analyze whether the expression of inflammatory and antiviral genes in respiratory syncytial virus (RSV)-infected infants' peripheral blood is associated with bronchiolitis progression.
Methods: We conducted a prospective study on 117 infants between 2015 and 2023. The expression levels of nine genes were quantified by quantitative polymerase chain reaction. Infants were classified according to their clinical evolution during hospital admission: (i) non-progression (n = 74), when the RSV bronchiolitis severity remained stable or improved; (ii) unfavorable progression (n = 43), when the RSV bronchiolitis severity increased. The association analysis was performed by logistic regression, adjusted by age, gender, prematurity, and RSV bronchiolitis severity in the emergency room.
Results: Infants were 57.3% male, and the median age of the study population was 61 days. Thirty-five infants (30.7%) were admitted to the intensive care unit after hospital admission. Univariate logistic models showed that tumor necrosis factor (*TNFα*) and chemokine (C-C motif) ligand (*CCL5*) gene expression at baseline were inversely associated with unfavorable progression, which was confirmed by multivariate analyses: *TNFα* (adjusted odds ratio = 0.8 [95% confidence interval = 0.64-0.99], *P*-value = 0.038) and *CCL5* (adjusted odds ratio = 0.76 [95% confidence interval = 0.62-0.93], *P*-value = 0.007).
Conclusions: An inadequate immune response to RSV, characterized by reduced gene expression levels of *CCL5* and *TNFα* in peripheral blood, was associated with an unfavorable progression of RSV bronchiolitis.

Introduction

Respiratory syncytial virus (RSV) infection in infants ranges from asymptomatic or mild upper respiratory infections to severe lower respiratory tract infections, primarily bronchiolitis, requiring hospitalization and intensive care [1]. RNAs and double-stranded RNA generated during RSV replication are recognized by retinoic-acid-inducible gene I and toll-like receptors, triggering intracellular signaling pathways that induce the expression of proinflammatory cytokines, chemokines, and interferons (IFN) [2]. IFN-mediated signaling upregulates the expression of numerous interferon-stimulated genes and establishes an antiviral state to restrict viral replication. However, an imbalanced immune response can lead to immunopathology [2]. Consequently, an inadequate immune response that fails to control RSV replication is associated with increased disease severity, while a robust innate immune

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response controls RSV spreading and decreases bronchiolitis severity [2]. Nevertheless, the underlying mechanisms contributing to RSV pathology remain unclear, and no robust biomarkers have been identified that accurately define who will develop severe RSV disease [3]. The early detection of high-risk infants can help improve disease management and reduce RSV infection-related sequelae.

We aimed to analyze whether the expression of inflammatory and antiviral genes in RSV-infected infants' peripheral blood is associated with RSV bronchiolitis progression.

Methods

We carried out a prospective study on 117 RSV-infected infants under the age of 2 years. The samples were collected between 2015 and 2023 in the Pediatric Service at Hospital Clínico de Valladolid, Hospital Infanta Cristina, and Hospital Universitario de Guadalajara, Spain. All patients tested positive for RSV by a polymerase chain reaction (PCR) test. The study was performed according to the Declaration of Helsinki. It was approved by the Ethics Committee of Instituto de Salud Carlos III (PI 84_2015-v2) and the Institutional Review Board of the respective hospitals. All infants' parents or legal guardians provided informed consent.

The Bronchiolitis score of Sant Joan de Déu (BROSJDD) and the Wood-Downes score (WDS) was used to calibrate the RSV bronchiolitis severity at baseline and during the follow-up. The BROSJDD range from 1 to 16, stratifying the population into mild (≤5), moderate (6-10), and severe (≥11) bronchiolitis. The WDS range from 1 to 10, stratifying the population into mild (≤3), moderate (4-7), and severe (≥8) bronchiolitis. Infants were classified according to their clinical evolution during hospital admission: (i) nonprogression, when the bronchiolitis severity remained stable (mild or moderate) or improved during follow-up (moderate to mild); (ii) unfavorable progression, when the bronchiolitis severity increased from baseline (mild to moderate, mild to severe, and moderate to severe).

Whole blood samples were obtained within the first 24 hours of admission to the emergency room and stored at −80°C. Total RNA was extracted using the NucleoSpin RNA Kit (Macherey-Nagel, Düren, Germany) and reverse transcribed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). The expression of the selected genes was quantified by real-time PCR (RT-PCR) using the TaqMan Gene Expres-

Table 1
Summary of characteristics of respiratory syncytial virus patients according to the progression of respiratory syncytial virus disease severity.

Characteristic	All	Nonprogression	Unfavorable progression	*P*
No. patients	117	74	43	
Age (days)	61 (30.5-137.2)	61 (30.5-152.5)	61 (28-91.5)	0.089
Gender (male)	67 (57.3%)	42 (56.8%)	25 (58.1%)	0.884
Medical history				
<6 weeks	46 (39.3%)	26 (35.1%)	20 (46.5%)	0.225
Weight (kg)	5.3 (4-6.9)	5.6 (4.4-8)	4.1 (3.6-5.7)	**0.001**
Breastfeeding	88 (80%)	55 (79.7%)	33 (80.5%)	0.921
Prematurity	4 (3.4%)	1 (1.4%)	3 (6.9%)	0.106
Family history of allergy, asthma, or atopy	26 (27.4%)	18 (31.0%)	8 (21.6%)	0.316
Clinic and admission				
The onset of respiratory symptoms (days)	3 (2-4)	3 (2-4)	3 (2-4)	0.176
rhinorrhea	113 (96.6%)	71 (95.9%)	42 (97.7%)	0.620
Dyspnea	78 (67.2%)	49 (66.2%)	29 (67.4%)	0.972
Acute otitis media	9 (7.8%)	6 (8.3%)	3 (6.9%)	0.793
Vomiting	40 (35.4%)	31 (43.7%)	9 (21.4%)	**0.017**
Feeding intolerance	31 (26.5%)	20 (27.0%)	11 (25.9%)	0.864
Respiratory frequency >60	44 (37.6%)	29 (39.2%)	15 (34.9%)	0.643
O_2 saturation <91	89 (76.1%)	59 (79.7 %)	30 (69.8%)	0.223
Laboratory findings				
Respiratory frequency	57 (50-62)	60 (50-62)	55 (46-64)	0.369
Cardiac frequency	155 (140-170)	153 (140-170)	158 (143-170)	0.398
Gasometry				
pH	7.4 (7.3-7.4)	7.4 (7.3-7.4)	7.3 (7.3-7.4)	0.288
PCO_2	47.1 (41-55)	45.9 (41-53.3)	51 (41.3-57.5)	0.256
PO_2	44.3 (37-55.2)	43.6 (37-54.2)	50.5 (38.2-58.7)	0.589
HCO_3	25 (23.1-27.4)	24.9 (23.2-27)	25.8 (22.4-29.1)	0.739
Blood count (x 10^3 cells/µl)				
Lymphocytes	47.4 (34.8-56)	48 (35.4-56)	46.5 (34.3-56)	0.548
Neutrophils	40 (30.8-52)	40.1 (31-53.1)	38.5 (29.1-49.6)	0.716
Mastocytes	9.3 (6.7-13.1)	9.3 (7.1-12.6)	9.5 (6.3-13.2)	0.940
Eosinophils	0.4 (0-1.2)	0.4 (0-1)	0.6 (0.1-1.6)	0.484
Basophils	0.2 (0-0.4)	0.2 (0-0.4)	0.3 (0.1-0.7)	0.534
Treatment				
Bronchodilator	63 (54.3%)	37 (50.7%)	26 (60.5%)	0.307
Beta-2 agonists	47 (40.1%)	28 (37.8%)	19 (44.2%)	0.499
Adrenaline	66 (56.4%)	40 (54.1%)	26 (60.5%)	0.500
Systemic corticoids	15 (12.8%)	10 (13.5%)	5 (11.6%)	0.769
Antibiotherapy	43 (36.8%)	26 (35.1%)	17 (39.5%)	0.634
Evolution and events				
Hospitalization days	7 (4-10)	7 (4-9)	7 (5-12)	0.106
O2 days	5 (3-8)	5 (2-7)	6 (4-10)	0.059
Noninvasive mechanical ventilation (days)	4 (0-6)	3.5 (0-6)	4 (0-8)	0.268
Pediatric intensive care unit	35 (30.7%)	16 (21.9%)	19 (46.3%)	**0.007**
Viral coinfection	45 (38.5%)	29 (39.2%)	16 (37.2%)	0.991

Statistics: Values are expressed as the median and interquartile range for continuous variables and absolute count (percentage) for categorical variables. The statistically significant differences are shown in bold.

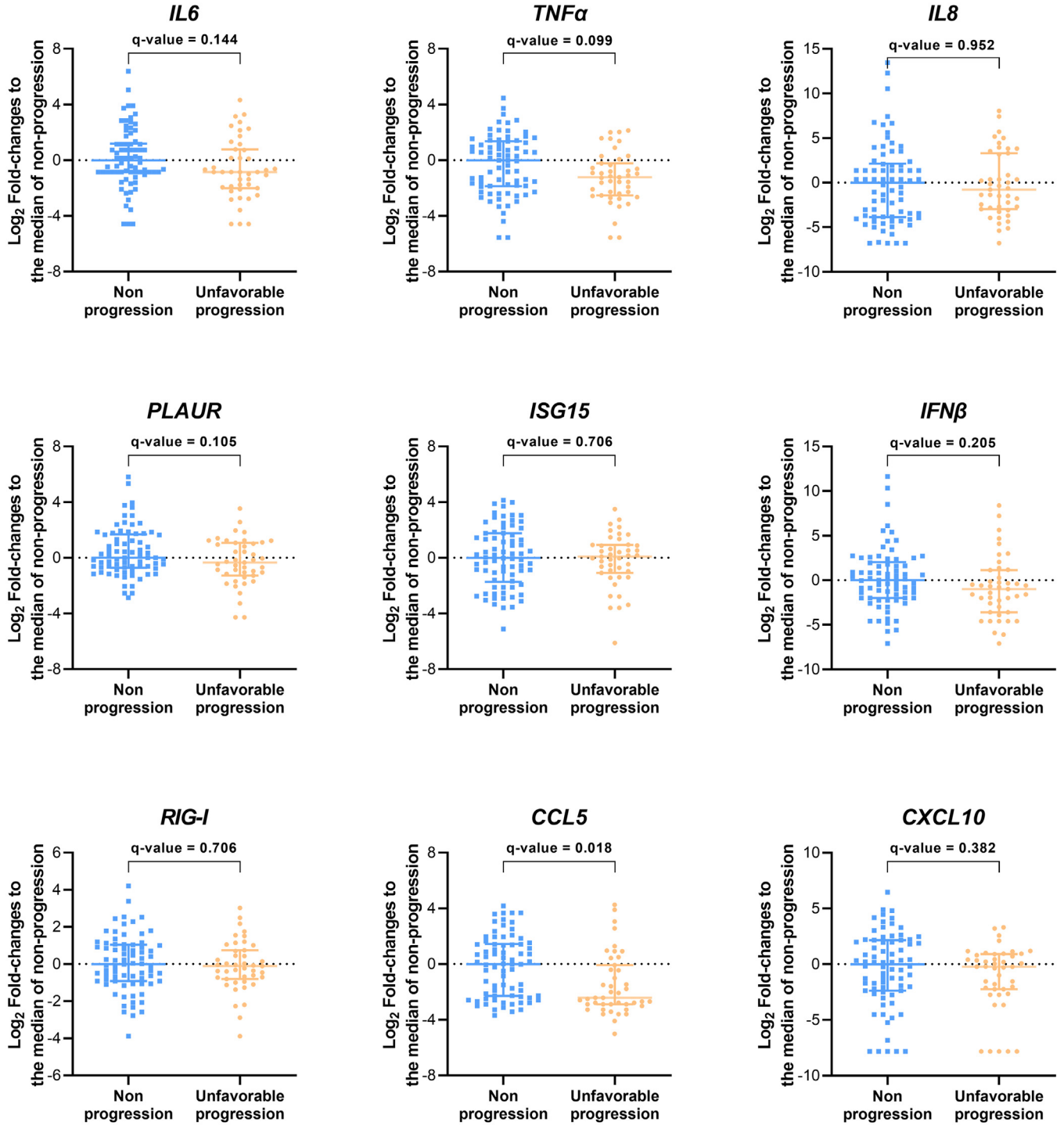

Figure 1. Gene expression of *IL6, TNFα, IL8, PLAUR, ISG15, IFNβ, RIG-I, CCL5*, and *CXCL10* in peripheral blood according to RSV bronchiolitis progression in infants during the follow-up (non-progression vs. unfavorable progression).
Statistics: Gene expression levels are expressed as a Log2 fold-change to the median of the non-progression group. The differences between groups were assessed using univariate logistic regression. *P*-values were adjusted by false discovery rate (q-value).
Abbreviations: IL-6, interleukin 6; TNFα, tumor necrosis factor α; IL-8, interleukin 8; PLAUR, plasminogen activator, urokinase receptor; ISG15, interferon-stimulated gene 15; IFN-β, interferon-beta; RIGI, retinoic acid-inducible gene I, CCL5, chemokine (C-C motif) ligand 5; CXCL10, C-X-C motif chemokine ligand 10.

sion Assays (Applied Biosystems, Foster City, CA, USA) described in Supplementary Table 1. PCR assays were performed in triplicate using a StepOne RT-PCR System thermal cycler (Applied Biosystems). Differential expression analysis was performed by the Ct (Cycle threshold) (ΔΔCT) method, using *ACTB* as endogenous control. Gene expression levels were determined relative to a calibrator from a reference sample of total RNA extracted from RSV-infected A549 cells.

Statistical analysis was performed using Stata IC 17 (StataCorp, Texas, USA). All *P*-values were two-tailed, and the significance level was set at 0.05. Mann-Whitney U-test for continuous variables and Pearson's chi-square test ($\chi 2$) or Fisher's exact test for categorical variables were used for between-group comparison. Binomial logistic regression was used to estimate the association between biomarkers at baseline and the unfavorable progression during follow-up, providing odds ratio (OR), 95% confidence

intervals (CIs), and *P*-values, which were corrected for multiple testing using the false discovery rate (q-value). Biomarkers with q-value <0.1 were selected for logistic regression analyses adjusted by the most significant covariables (age, gender, prematurity, and baseline RSV bronchiolitis severity), which were selected by a stepwise forward selection method (pin <0.05 and pout <0.10).

Results

Infants were 57.3% male, and the median age was 61 days. The main symptoms observed were rhinorrhea, dyspnea, acute otitis media, vomiting, and feeding intolerance. A total of 35 infants (30.7%) were admitted to the pediatric intensive care unit (PICU) after hospital admission (Table 1). Infants who did not progress had a higher frequency of vomiting, greater weight, and lower frequency of PICU admission than those with unfavorable progression (P <0.05).

At the time of hospital admission, 41 infants had mild bronchiolitis, while 76 had moderate bronchiolitis. There were no significant differences in gene expression values between the groups (Supplementary Figure 1; q-value<0.1).

During follow-up, 64.3% (27/42) of infants with mild bronchiolitis and 20.8% (16/77) with moderate bronchiolitis exhibited unfavorable progression. Those with an unfavorable progression showed lower tumor necrosis factor (*TNFα*) and chemokine (C-C motif) ligand 5 (*CCL5*) expression levels at baseline than those with stable clinical course (Figure 1; q-value<0.1). These findings were further confirmed by multivariate logistic regression: *TNFα* (adjusted OR = 0.8 [95% CI = 0.64-0.99], *P*-value = 0.038) and CCL5 (adjusted OR = 0.76 [95% CI = 0.62-0.93], *P*-value = 0.007).

Discussion

The host's immune response against RSV is crucial in controlling and clearing the infection. Our study showed that low gene expression of *CCL5* and *TNFα* in peripheral blood mononuclear cells was associated with unfavorable progression of RSV bronchiolitis in infants. Peripheral blood can serve as a liquid biopsy for studying lung immunity against viral infections and for identifying predictive biomarkers, owing to the close connection between the circulatory and respiratory systems [4]. Furthermore, it provides valuable information about the systemic immune response triggered by the infection.

CCL5 is a chemokine that plays a pivotal role in the host immune response by attracting monocytes, T cells, neutrophils, and eosinophils. It also promotes macrophage survival, which is essential for virus control [5]. In line with this, other studies found an inverse correlation between CCL5 levels in the tracheal aspirate and nasal fluid samples and the severity of RSV infection [6]. These findings suggest that reduced CCL5 levels might significantly impact the immune systems' capacity to recruit and activate inflammatory cells necessary for controlling RSV infection [7]. Furthermore, it has been postulated that CCL5 exhibits a direct antiviral effect on RSV by inhibiting the interaction between RSV fusion protein and epithelial cells [8]. Our findings have also been corroborated in SARS-CoV-2 infection, where low CCL5 levels have been linked to disease severity [9].

TNFα is a proinflammatory cytokine that plays a crucial role in the host response to infection by mediating immune and inflammatory responses and inducing autocrine expression of other cytokines, including interleukin (IL)6 and chemokines such as CCL5 [2]. Previous studies have demonstrated that TNFα acts as a protective factor against RSV infection, consistent with our findings, where patients who maintained a stable clinical course exhibited higher levels of TNFα and IL6 upon admission [10]. Moreover, low levels of TNFα and IL6 increased the likelihood of hospitalization [11].

CCL5 and *TNFα* gene expression could serve as potential predictive biomarkers in the clinical setting, using a qPCR assay that is easily applicable in the hospital environment. However, it is essential to note that our study should be considered preliminary. In this context, the *CCL5* and *TNFα* gene expression levels significantly differed between the groups. However, there was a partial overlap, which makes it difficult to consider them as biomarkers with high predictive performance. Furthermore, our study has several limitations, including evaluating a limited number of biomarkers and a small sample size. Consequently, further studies are warranted to validate our findings.

In conclusion, CCL5 and TNFα seem to be essential components of an immune response that effectively controls the progression of RSV infection since reduced gene expression levels of *CCL5* and *TNFα* in peripheral blood were associated with an unfavorable outcome. These genes are promising biomarkers of the clinical course of RSV bronchiolitis and provide valuable information on the immunopathology of RSV infection.

Declarations of Competing Interest

The authors have no competing interests to declare.

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Author contributions

Funding acquisition: SR. Study concept and design: SR and IM. Patients' selection and clinical data acquisition: CGS, AGP, EMM, MRM, and VM. Sample preparation, RNA isolation, and RT-PCRs: CPM, AVV, and RAS. Statistical analysis and interpretation of data: CPM, AVV, and SR. Writing – original draft preparation: CPM, SR, and IM. Writing – Review & Editing: CGS, AVV, and VM. Supervision and visualization: SR and IM.

Availability of data and materials

The datasets used and analyzed during the current study are available from the corresponding authors upon reasoned request.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ijid.2023.11.024.

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