Nasopharyngeal and peripheral blood type II Interferon signature evaluation in infants during respiratory syncytial virus infection.

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Short Title: Type II IFN signature in RSV infection

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Abstract

Background: In this study, we applied one-step real time rt-PCR technology type II INF signature to blood and nasopharyngeal (NPS) swabs of acute early recovery children <1 years hospitalized for bronchiolitis with laboratory-confirmed RSV infection.

Methods: A prospective observational case-control study was conducted in 2021–2022. The study took place in Children Hospital " Regina Margherita", Torino Italy. The study included 66 infants, of which 30 patients hospitalized

for bronchiolitis due to RSV infection and 36 age matched controls. Inclusion criteria included a positive RSV test for infant with bronchiolitis. We collected peripheral blood and nasopharyngeal swab for relative quantification of type II Interferon signature by One-Step Multiplex PCR real time.

Results: IFN levels were downregulated in the peripheral blood of bronchiolitis patients, these data were not confirmed in the nasopharyngeal swab. There was no correlation between NPS and the type II IFN score in peripheral blood.

Conclusion: our study shows for the first time that type II IFN score was significant reduced in peripheral blood of infants with bronchiolitis by RSV compared to age matched healthy controls; in the NPS swab this downregulation resulted not statistically significant and type II IFN score in NPS swab can be used as marker of resolution of infection or improvement of clinical conditions.

Key words: Type II IFN signature, IFNγ, RSV, infants , PCR real time.

1 INTRODUCTION

Respiratory syncytial virus (RSV) is the main cause of hospitalization for bronchiolitis among infants younger than 12 months [1]. Worldwide, RSV causes almost 34 million lower respiratory tract infections (LRTI) with an estimated annual increase of 10% and 3.4 million hospitalizations per year in infants and children less than 5 years of age. [2, 3]. In numerous nations, including Italy, RSV currently represents a public health issue [4]. RSV belongs to the Pneumoviridae family [5] and is characterized by a large envelope and a negative-sense RNA (approximately 15-16 kb) encoding for 11 proteins, which include both non-structural and structural proteins. It owes its name to its ability to produce syncytia from adjacent cells in the host following infection with the virus. It is an RNA virus with a linear single-stranded genome surrounded by a helical nucleocapsid and this in turn by a lipoprotein envelope giving it a spherical or filamentous appearance. Prominent among the latter are the membrane glycoproteins G and F which mediate, respectively, adhesion and fusion to the host respiratory tract epithelial cell surface. Glycoprotein F is also involved in the formation of the characteristic syncytia. Structural proteins are also the matrix protein (M), involved in virus assembly, two nucleocapsid proteins (N and P) and M2-1 and M2-2 proteins, responsible for transcriptional activity and regulation. RSV also presents an RNA-dependent RNA polymerase (L) that regulates transcription and replication of the virus in the cytoplasm of the host cell once penetration has occurred. The non-structural proteins NS1 and NS2 are the first to be transcribed during infection, interfering with the interferon (IFN) response and other elements of the immune system. RSV infections show seasonality, with peaks through the winter months in temperate

regions [6,7]. The elderly, young children, and those with chronic medical conditions are at the highest risk for severe RSV infections [8,9]. The initial RSV infection progresses, affecting the lower respiratory tract and in 2-3 days 25-30% of children develop acute bronchiolitis. The initial picture of rhinitis and cough evolves to continuous cough, progressive increase of respiratory work, intense decay and refusal of food. Clinical signs (tachypnea, tugging, nasal flaring, disseminated wheezing, thoracic hyperinflation, generalized hypoventilation, hypoxemia and cyanosis) and radiological signs (air trapping, areas of consolidation or major complications such as pneumonia and atelectasis) characteristic of severe bronchopulmonary involvement stand out in the examination. The evolution of acute bronchiolitis is unpredictable when the disease begins: most children with acute RSV bronchiolitis, previously healthy and without pulmonary complications, improve in 3-4 days without requiring hospitalization; of those hospitalized, many improve with symptomatic treatment, oxygen therapy, and can be discharged in 2-3 days. On the other hand, 1-3% of the youngest infants (under 6 months, especially under 2 months) and children with underlying conditions usually develop pulmonary complications. They require longer hospitalization, often admission to the pediatric or neonatal ICU for respiratory support and treatment of the respiratory complications they develop (pneumonia, pneumothorax, atelectasis. However, there is some evidence that the ability to develop an adequate type I-like immune response during primary RSV infection is impaired in the development of severe lower respiratory tract disease [10].

Although IFN type II, known as IFN-γ, has a similar nomenclature to IFN type I, it is signaled via a different receptor, has effects that are independent of IFN type I and are mainly produced by natural killer cells during infection (2 Originally identified 30 years ago as an agent with antiviral activity, IFN-γ has since been characterized as a homodimeric glycoprotein with pleiotropic immunologic functions. IFN-γ is primarily secreted by activated T cells and natural killer (NK) cells, and can promote macrophage activation, mediate antiviral and antibacterial immunity, enhance antigen presentation, orchestrate activation of the innate immune system, coordinate lymphocyte–endothelium interaction, regulate Th1/Th2 balance, and control cellular proliferation and apoptosis. It was not until 20 years after the identification of IFN-γ that its cell-surface receptor was discovered. The α chain of the IFN-γR, also known as IFN-γR1 or CD119, was the first component of the receptor to be identified and cloned. Although it binds IFN-γ with relatively high affinity, IFN-γR1 alone is unable to mediate the biologic responses to this cytokine. Subsequent complementation studies led to the identification and cloning of an accessory factor (AF-1), also known as the β receptor chain or IFN-γR2, as the protein required, in addition to IFN-γR1, to endow a cell with the ability to respond to IFN-γ. Specific residues within the cytoplasmic domains of both the α and β chains of the IFN-γR are critical for transducing the IFN-γ signal from the cell surface to the nucleus through the activation of intracellular signaling pathways. [11-13]. The IFN-γ is the sole IFN released by NK cells and mostly by Th1 cells, which also elicits activation of thousands of genes [14].

CXCL9, CXCL10, and IDO1 are prevalently IFN-γ-stimulated genes (ISG). Their expressions correlate with the tissue infiltration of inflammatory cells, in particular of T cells [15]. Downregulation of IFN-γ were reported in children with Influenza Virus illness, SARS-CoV2 and RSV bronchiolitis [16-18]. IFN-γ has immune-regulatory functions that work to optimize the antiviral response and limit exaggerated responses that could lead to collateral damage. An optimal antiviral response involves both activating beneficial immune responses, while simultaneously inhibiting impractical and potentially damaging responses [19].

Several RSV transcriptome studies have been performed using in vitro models [20-26], animal models [27–31] and human subjects [32–38]. However, most in vivo studies only investigated systemic transcriptional profiles in blood [33–35, 37, 38]. Only one study investigated local respiratory expression profiles by analyzing nasopharyngeal swabs (NPS) from hospitalized children (n = 30) [39].

In this study, we applied one-step real time rt-PCR technology type II INF signature to blood and nasopharyngeal (NPS) swabs of acute early recovery children <1 years hospitalized for bronchiolitis with laboratory-confirmed RSV infection.

2 RESULTS

2.1 Study populations

At enrollment all the patients were screened for the RSV infection using rapid Antigen Xpert Xpress FLU/RSV . (Cepheid, Sunnyvale, CA, U.S.A.): 30 infants suffering of bronchiolitis were positive for RSV infections and 36 healthy controls were negative. No coinfection were detected.

White blood cells count (WCC) were higher in the RSV group, but not significantly. However, lymphocytes and monocytes were significantly higher in the RSV group than the healthy controls (Table 1).

Table 1. Characteristics of the study population in terms of age and White blood cells count

Variable	Bronchiolitis (30)*	Healthy Controls (36)*	pValue§
Age (days)	86±84	92±101	0.585
White blood cells count (cells x 109/L)	9670±650	8830±992	0.386
Neutrophils (cells x 109/L)	2834±1129	3228±1010	0.545

Lymphocytes (cells x 109/L)	5400±1998	4180±1883	0.032°
Eosinophils (cells x 109/L)	302±100	443±80	0.310
Monocytes (cells x 109/L)	1503±1030	632±301	<0.0001°

*Data are reported as mean and SD.

§Mann-Whitney U test.

2.2 *IFN-Stimulated Genes Expression Evaluated by qPCR*

As a first attempt, we investigated type II IFN signature in blood samples. We calculate IFN score for each samples as show in figure 1. The IFN score were significantly higher in HC than in bronchiolitis subject. Mean value: IFN score RSV positive bronchiolitis samples: 1.14±1.25 vs Mean value: IFN score Healthy controls 2.41±2.14 ($p < 0001$).

Figure 1. Statistical analysis: Mann-Whitney test was used to compare IFN score in the peripheral blood of bronchiolitis RSV patients vs healthy control. Circles and squares show IFN score of bronchiolitis and healthy controls, horizontal lines the median values.

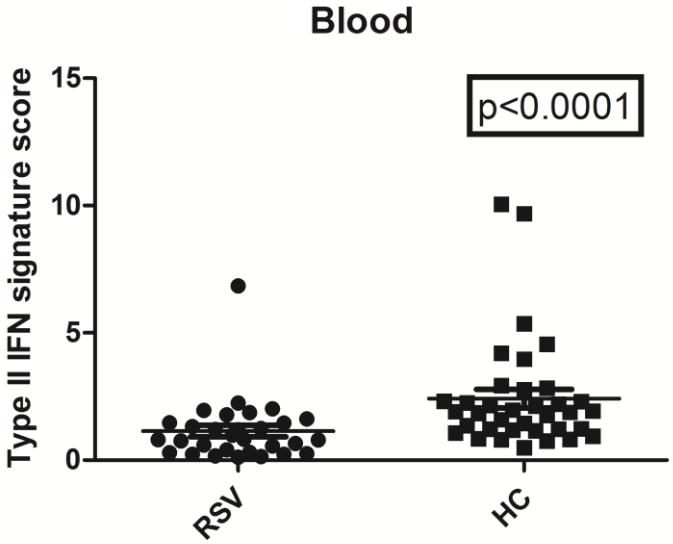


Table 2. ΔΔCt data and Interferon score obtained in blood of bronchiolitis RSV positive subjects.

CXCL9	CXCL10	Ido1	IFN	IFN score
0.157065	8.18543	0.286978	0.164163	0.22557
0.624713	0.679134	0.553085	0.197167	0.588899
0.061826	0.16016	0.069976	0.311058	0.115068
0.931124	2.576414	1.962246	0.549406	1.446685
0.587774	2.936548	4.420878	0.804047	1.870297
1.766501	3.628207	1.800975	0.693805	1.783738
0.302649	1.540725	1.108899	2.195808	1.324812
0.58293	1.376756	1.961242	0.326026	0.979843
0.937127	35.02627	1.534166	0.776836	1.235647
0.728682	143.6117	12.19011	1.500456	6.845283
0.09159	25.39361	2.204072	0.203047	1.203559
0.660717	51.14568	3.818783	0,229422	2.23975
0.068105	0.242614	0.109202	0.234683	0.171942
0.127123	0.490703	0.448894	0.170006	0.30945
0.457043	9.124063	0.856635	0.262696	0.656839
1.638153	6.903458	0.736292	0.049596	1.187223
2.700755	6.703653	0.173626	1.332774	2.016765

0.282434	0.369647	0.176321	0.295971	0.289203
0.470063	14.85163	1.255824	1.693257	1.474541
0.802751	270.6202	3.105014	0.606671	1.953883
0.229872	3.890983	0.424244	0.382459	0.403351
0.693212	4.8035	0.830444	0.321862	0.761828
0.159718	0.358076	0.063926	0.44009	0.258897
0.372456	1.831377	0.710723	0.885488	0.798105
1.423442	1.934601	0.320828	1.820754	1.622098
0.276544	1.094884	0.69411	0.886863	0.790487
0.189038	0.331033	0.059418	0.266275	0.227656
0.060295	0.15324	0.119145	0.968841	0.136193
0.174432	0.771439	0.319235	0.974291	0.545337
0.45667	13.39745	0.651593	0.98962	0.820607

122

123 **Table 3**. ΔΔCt data and Interferon score obtained in blood of bronchiolitis RSV positive subjects.

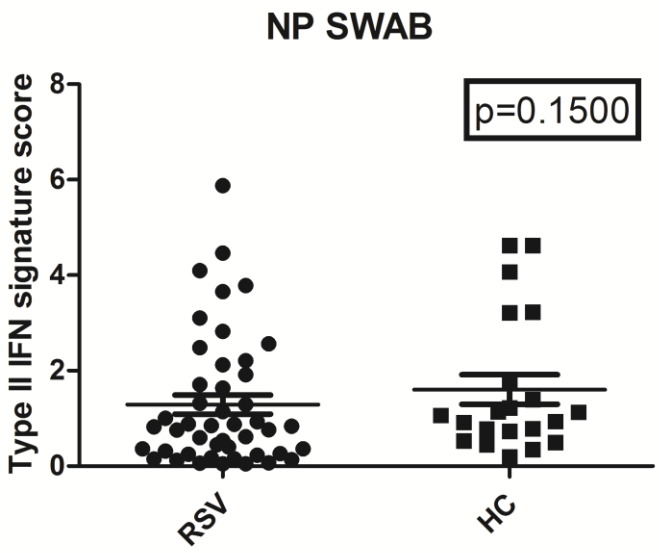
CXCL9	CXCL10	Ido1	IFN	IFN score
0.927221	5.927591	2.814915	1.059956	1.937436
0.807148	0.578726	1.007283	0.800781	0.803965

0.961704	2.670438	0.346059	0.938156	0.94993
0.553646	3.473331	2.624912	1.69073	2.157821
0.558547	3.529864	1.485558	0.880558	1.183058
1.166531	6.642831	3.234286	1.094232	2.200409
0.574403	0.930456	0.491075	0.977094	0.752429
0.703173	3.167205	2.206281	1.306828	1.756554
0.593752	2.681653	2.472985	1.178443	1.825714
0.526364	2.241326	2.699156	0.94144	1.591383
0.789334	16.15611	1,675641	0.349211	1.232488
0.449238	1.028112	1.120321	2.891309	1.074216
3.026584	33.28678	3.15599	4.782118	3.969054
1.415051	6.074084	2.684512	1.954509	2.31951
1.233968	5.30949	3.012727	2.855546	2.934137
1.818892	54.26082	2.671744	1.829073	2.250408
1.482601	7.572696	3.070401	5.337788	4.204095
0.198741	1.123516	7.019939	1.755256	1.439386
0.246297	2.135114	2.707466	1.617702	1.876408
0.315997	3.798261	1.361492	0.123542	0.838745
0.547174	2.802106	3.099739	2.73358	2.767843
0.299992	31.17147	14.73604	5.374277	10.05516
0.62112	1.344691	3.800596	1.08666	1.215676

0.927419	12.62865	9.230163	1.47954	5.354851
0.525293	3.374039	2.958201	2.699701	2.828951
0.473346	7.375175	1.825008	1.997246	1.911127
0.831074	5.225671	0.523769	1.607206	1.21914
0.667976	1.468509	0.821468	0.810344	0.815906
1.640676	11.53084	1.537516	2.962262	2.301469
4.472612	44.21708	10.61164	8.757177	9.684408
0.422769	1.28918	1.027396	2.246802	1.158288
0.554591	2.148308	2.335841	0.387631	1.35145
4.242832	20.40956	2.606556	4.880706	4.561769
0.927105	3.324605	0.747185	3.002083	1.964594
1.444508	2.796676	1.462326	3.054695	2.129501
0.192616	0.748958	0.230515	1.446799	0.489737

As a second attempt, we investigated type II IFN signature in NPS swab samples. We calculate IFN score for each samples as show in figure 2. The IFN score were higher but not statistically significant in HC than in bronchiolitis subject. Mean value: IFN score RSV positive bronchiolitis samples: 1.29±1.37 vs Mean value: IFN score Healthy controls 1.55±1.45 ($p = 0.15$).

Figure 2. Statistical analysis: Mann-Whitney test was used to compare IFN score analyzed in NPS swab of bronchiolitis RSV patients vs healthy control. Circles and squares show IFN score of bronchiolitis and healthy controls, horizontal lines the median values.



We calculate IFN score correlation analysis between NPS and blood and not try statistically significant with p=0.8 (figure 3).

Table 4. ΔΔCt data and Interferon score obtained in NPS of bronchiolitis RSV positive subjects

IFNg	CXCL9	CXCL10	IDO1	IFNscore
1.685659	0.106575	0.060205	0.190854	0.177204
0.199817	0.450834	0.357298	0.520083	0.450208
0.6063	0.114727	0.115976	2.218294	0.847529
2.364469	1.466056	1.387023	3.230586	1.915262
1.629892	0.515913	0.334127	1.168483	0.884869
2.218103	0.494326	0.552003	2.591719	2.211611
0.742426	3.30203	7.521585	61.60443	5.878039
0.288555	0.349889	0.12762	0.980016	0,319222
0.937276	0.708254	0.318651	3.882854	0.822765
2.629424	3.740852	2.294862	25.64459	4.465884

8.459833	0.68337	2.678824	24.66566	3.105226
0.116979	0.333878	0.191974	0.371062	0.262926
1.939566	1.338197	0.955549	12.77733	1.638882
4.688201	0.820236	1.056642	30.713	3.659446
//	0.711009	0.875694	7.346527	2.825088
0.042141	0.051571	0.025111	0.01295	0.046856
0.357725	0.264255	0.155025	3.177838	0.36583
2.861291	1.714959	2.135358	14.25268	2.485898
2.379219	0.3004	0.203204	2.950032	0.754847
0.989339	2.618225	0.873043	3.202769	2.563137
0.05656	0.025731	0.016338	0.130882	0.062945
0.590727	1.039541	0.494817	1.226243	0.764687
//	1.674171	0.778514	2.732303	1.142535
1.114745	0.356293	1.091995	18.2477	1.293056
3.358902	1.725384	1.169357	2.415619	2.123589
//	//	//	7.065799	3.783915
0.033014	0.017777	0.028004	2.806203	0.071856
3.286032	1.858926	9.757487	45.052	4.096859
//	//	//	2.414923	1.316748
//	0.060658	0.041742	//	0.052629
//	0.159294	0.03838	1.285106	0.159294

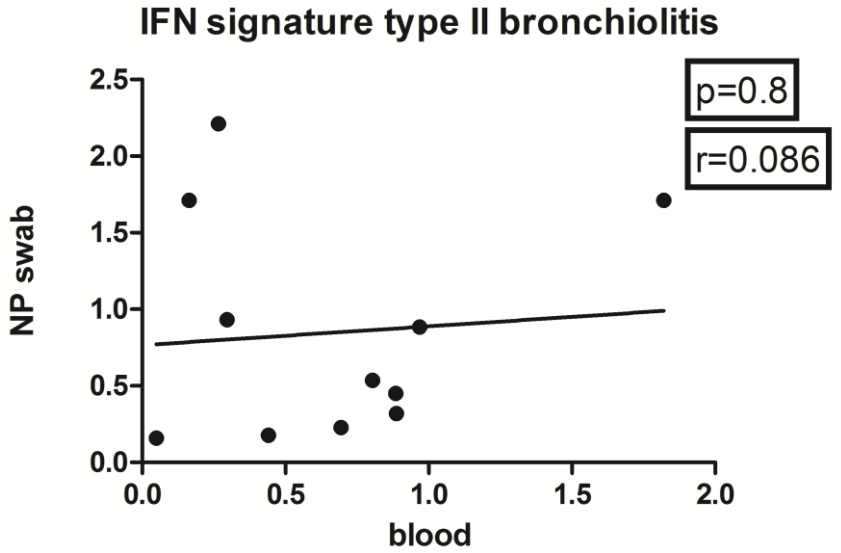
2.72743	1.535855	0.419061	1.133716	0.93273
1.111062	0.075519	0.066413	//	0.252402
0.228895	0.020628	0.01378	1.211771	0.148608
0.772693	0.185458	//	0.599208	0.599208
1.345381	0.09437	0.119168	//	0.125718
3.47402	0.311783	0.234453	2.285236	0.536745
//	1.710044	0.532687	1.712725	1.711384
//	//	0.124275	4.711086	0.617166
//	//	0.164969	4.244882	0.364803
//	0.272361	1.167929	6.361056	0.878366
//	0.672684	0.285985	0.403993	0.403993
//	//	0.262111	//	0.229
//	//	0.08179	//	0.134591
1.749853	0.583862	0.332689	0.660082	0.83433

Table 5. ΔΔCt data and Interferon score obtained in NPS of healthy subjects

IFNg	CXCL9	CXCL10	IDO1	IFNscore
0.119396	0.365331	0.212938	0.454007	0.351945
//	//	//	4.354237	3.21977
2.396478	3.402945	11.74381	21.757	4.622528
1.084336	0.501719	0.324502	0.174045	0.396846

1.370674	1.186268	0.581905	0.50408	0.732686
1.941057	1.744747	0.761611	0.479918	0.777233
0.900624	1.081239	0.490691	0.45876	0.530976
//	0.067341	0.494851	//	0.494851
0.570475	0.233015	0.373023	0.449391	0.449391
//	6.473846	7.796893	//	4.625882
4.727643	4.158529	2.109328	//	3.227281
0.478495	0.471916	1.254819	3.87319	0.919472
0.12972	0.0641	0.110914	2.88078	0.199274
3.129895	1.368325	1.13171	1.313043	1.222377
0.485004	1.065	0.874331	1.552961	1.132574
0.78607	1.009494	0.807926	0.295926	0.7852
1.857269	1.117105	0.639395	0.215011	0.936784
0.320975	1.104835	3.47792	3.986919	1.145811
0.84222	1.283846	0.833621	1.326317	1.063033
1.786934	3.155041	2.117417	0.710685	1.805562
7.505436	7.622924	4.274014	0.492337	4.072474

Figure 3. Spearman correlation test was used to compare IFN score analyzed in NPS swab and peripheral blood of bronchiolitis patients.



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147 **3 DISCUSSION**

148 RSV is, in infancy, the most important etiological agent of acute lower respiratory tract infections, and the leading cause
149 of hospitalization in childhood, which results in a great problem on global health-care services as well as an important
150 pathogen for the elderly [40]. It is estimated that 50 % of children are infected with RSV in the first year of life, and
151 even 100 % of children under 3 years of age [41,42]. This effect is due to an incomplete protective immune response of
152 the host against RSV, as the virus can impair the development of effector and memory CD8+ T cells in the lung [43-
153 45].

154 IFN-γ has significant antiviral activity and is associated with the modulation of Th1- or Th2-like immune responses, as
155 IFN-γ influences the differentiation of naïve T cells into Th1 or Th2 cells [46]. Reduced interferon-γ cytokine levels
156 have been detected in airway samples from infants with severe RSV disease [47]. Similarly, a negative correlation was
157 found between IFN-γ mRNA levels and severity of RSV disease in nasopharyngeal samples [48], indicating a
158 suppressed type II IFN (IFN-γ) response. In blood, the data are conflicting, with several studies finding either a positive
159 association, a negative association or no association [49].

160 We performed for the first time an IFN score analysis for the type II IFN signature in NPS and peripheral blood. We
161 found a statistically significant decrease of type II IFN score in the peripheral blood of infants with RSV bronchiolitis.
162 We do not attempt to find a statistical difference in the NPS, although the same trend show in the blood is maintained.
163 We have not shown a correlation between the type II IFN score measured in the NPS and in the peripheral blood,
164 whereas Lopez and colleagues have recently demonstrated a correlation between IFN typeI/III in patients with

SARSCov2 infection [50]. This is probably related to the fact that IFN-I and IFN-III are involved in the first line of defense against infections against IFN type-II [51]. All infants studied were hospitalized with a first LRTI, were of similar age, had no family history of atopy and were therefore comparable to healthy controls. Several studies have reported a reduction in IFN type II plasma level in patients with severe COVID-19 and RSV infecton, which is comparable to our results [18,52] indicating a suppressed type II IFN (IFN-γ) response. A number of immune cell analyzes suggest that IFN-γ-producing CD4+T, CD8+T and NK cells are depleted in patients with severe COVID-19 [53, 54], which could plausibly explain the reduced IFN-γ plasma levels in ICU patients. A deficient type I immune response may also result from the immaturity of an infant's immune system. It is known that the competence to generate responses in infancy is regulated developmentally, and that the maturation process of IFNg production is heterogeneous in the normal population. The importance of age at first RSV infection in determining the subsequent pattern of T-cell responses upon reinfection has recently been demonstrated in a mouse model. Neonatal RSV infection resulted in more severe disease and strong type II cytokine responses upon reinfection, whereas delayed RSV infection resulted in increased production and less severe disease upon reinfection. These results suggest that early neonatal RSV infection may induce a long-lasting tendency for type II immune responses upon reinfection, emphasizing the importance of early infections in determining subsequent disease progression [52]. Joshi et al. showed that absolute levels of IFN-γ mRNA levels were also lower compared to infants in whom another virus or no virus was identified as the cause of respiratory symptoms. This suggests a suppression of Th1 cytokine responses at the airway level during RSV infections [55]. It would also suggest that other viral infections, particularly rhinovirus, upregulate production during acute infections. Lower levels of IFN-γ mRNA measured during RSV infection could favor the development of asthma: Renzi et al [27] found that in children hospitalized with acute RSV infection, those who developed asthma had significantly lower levels of IFNg produced by their PBMCs at the time of acute RSV infection than those who did not have asthma [56].

The discrepancy between the data obtained in blood and NPS is probably due to the nature of the biological sample. In the case of IFN type II, the NPS swab (mucosal defenses) returns to normal expression before the peripheral blood. In fact, all the infants tested recovered positively from the infections a few days later. The type II IFN value measured in the NPS could be used as a biomarker indicator for the severity of the infections, a rapid return to normal levels expression of type II ISGs show the end of diseases. Studies of cytokine response have shown conflicting evidence, probably due to marked heterogeneity in study design and sample size. Although the data suggest a predominantly decreased IFN-γ production in nasal samples, in blood the data are conflicting with either positive association [57], negative association [52], or a lack of association in several studies [49]. Compared to the type II IFN score measured in

peripheral blood, the score measured on an NPS result not invasive and could be reflects the real stage of infection as a marker for resolution of RSV infection in the individual infant. This biomarker is even more useful considering the fact that sampling in the case of a viral infection is often difficult to identify and standardize. How many days ago did the symptoms begin? When did infants access to the hospital? It is difficult to standardize the time of blood or swab sampling and testing.

However, the temporal sequence of events (low IFN-g levels predisposing to RSV infection and/or RSV infection worsening the ongoing immune response) in the individual infant can only be determined by a prospective study, which, due to the low incidence of severe LRTI in infants, would need to include a considerable number of newborns for the results to be sufficiently meaningful. The useful of NPS type II IFN score as a rapid marker for resolution of RSV infection in the individual infant can only be determined by a prospective study, which would need to include a substantial number of newborns for the results to be sufficiently meaningful due to the low incidence of severe LRTI in infants. A strong stimulus for IFN-g production may also be important for the maturation of cellular immune functions and for promoting the development of Th-1 cells in the first months of life. An infant's history of infection, rather than age, may therefore have a significant impact on the clinical development of respiratory disease, particularly with regard to the so-called sensitization phase in infancy.

Although the results shown in this paper are promising for improving the clinical management of bronchiolitis must be interpreted with caution. In conclusion, our study shows for the first time that type II IFN score was significantly reduced in peripheral blood of infants with bronchiolitis by RSV compared to age matched healthy controls; in the NPS swab this downregulation resulted not statistically significant and type II IFN score in NPS swab can be used as marker of resolution of infection or improvement of clinical conditions.

4 MATERIALS AND METHODS

4.1 Patients

This prospective study was conducted in Turin, Italy, between October 2022 and February 2023. We enrolled in the study full-term infants who were hospitalized in Early Infancy Special Care Unit of the Regina Margherita Children Hospital, Turin, Italy, for their first episode of bronchiolitis. The controls were healthy full-term infants below 12 months of age who attended an outpatient clinic at the Department of Paediatrics for routine postnatal checks.

Bronchiolitis was diagnosed by using clinical signs that included rales, wheezing with or without a cough, dyspnea and retractions of the respiratory muscles and increased respiratory rate. The hospitalized infants with bronchiolitis underwent routine blood and swab tests at their recovery in Hospital.

The parents of the enrolled infants (subject and control) were informed about the purpose, benefits and possible risks of the study and written, informed consent was obtained. The protocol was approved by the Ethics Committee of the Azienda Ospedaliera Città della Salute e della Scienza di Turin, Italy.

The mean age of the 30 bronchiolitis patients (46% male and 54% female) was 86 days (10-351) when they were admitted to hospital. Their mean gestational age at birth was 38 weeks and their mean birthweight was 3150 grams (2690- 3910) . The 36 infants in the control group (51.6% boys) were seen at a mean age of 92 days (22-333) and 43.2% were still being exclusively or predominantly breastfed. They had not been hospitalized for bronchiolitis or any other infections. In the 30 bronchiolitis subject mean gestational age at birth was 38 weeks and their mean birthweight was 3020 grams (2500 – 3880). White blood cells, neutrophils and eosinophils and RSV positivity were recovered from the medical records. All the samples were screened for other respiratory virus with Allplex Respiratory Panel Assays (Seegene Inc. Taewon Building, Seoul) and resulted negative.

In brief at admission (acute phase) blood was collected in ethylenediaminetetraacetic acid (EDTA), and RNApro (BioMole) and Nasopharyngeal swabs (NPS) (Copan Diagnostics Inc, Murrieta, CA) were collected in RNApro (BioMole). NPS and blood were processed for host gene expression profile analyses. NPS recovered from healthy subject were 20 out of 30.

The exclusion criteria for the patients and controls included known or suspected impairment of immune function, congenital malformations and premature births under 37 weeks' gestation. A paediatric investigator recorded the personal data provided by the parents or guardians and the clinical data collected during the physical examinations.

The study and the data collection procedure were approved by the Ethics and Research Committee of the Città della Salute e della scienza di Torino on 24.11.2014 prot. Verbal informed consent was obtained from the parents of the study participants and recorded in the medical records in accordance with the Italian guidelines for good clinical practice and clinical investigations. The samples were anonymized before processing.

4.2 mRNA isolation and Real-time PCR

For each nasopharyngeal swabs and peripheral blood, RNA were extracted using simply RNA Blood Kit protocol in Maxwell16 system (Promega, Madison, WI), according to the manufacturer's instructions. Prior to extraction, swabs

and peripheral blood were maintained in RNApro (BioMole), a stabilizer that permit conservation of the samples in -80°C until use without RNA degradation. RNA was eluted in a final volume of 50 µL. RNA purity and concentration were evaluated by spectrophotometry using Simplinano (Biochrom, Cambridge, UK). 260/280 Absorbance ratios was used to assess the purity of nucleic acid extracted.

Relative quantification of type II IFN signature was achieved by IFNsig. Type II One-Step Multiplex PCR real time kit cod. BM-024 (BioMole, Turin, IT). Amplification were run in CFX96 Real-Time System (Bio-Rad Laboratories, Segrate Milan, IT) using Maestro software ver. 1.0. The IFNsig.Type II Multiplex One Step kit is a real-time PCR assay able to reverse-transcribe and amplify RNA in a single step. Thanks to the multiplex version, the RNA is tested with two different mixes that will return the data of the genes stimulated by Interferon γ and the housekeeping gene.

10 ng of RNA were amplified in a 20 µl total volume reaction. The amplifications were performed in a 96-well plate at 50°C for 10 min, 95 °C for 10 min, followed by 40 cycles of 95 °C for 10 s and 60°C for 30 sec for a total time of 80 min.

4.3 IFN signature analysis

The expression of six IFN-stimulated genes was assessed by qPCR using CFX96 Real Time PCR System (BioRad) and IFNsig. Type II One-Step Multiplex PCR real time kit (BioMole) for IFN-γ, CXCL9, CXCL10 and IDO1, [15]. Each target quantity was normalized with the expression level of GAPDH, and the relative quantification (RQ) was conducted relating to a "calibrator" sample (mix of 30 healthy controls) using the $2^{-\Delta\Delta Ct}$ method [58]. The median fold change of the six genes was used to calculate the IFN score for each subject.

4.4 Statistical analysis

Statistical analyses were performed using GraphPAD Prism5 (GraphPad Software, La Jolla, CA, USA). We used the non-parametrical Mann Whitney U-Test to compare IFN- score in the analyzed patient groups and controls. We used Spearman correlation test comparing IFN score between NPS swab and blood. We considered a significant difference if the p value was <0.05.

Author Contributions

All authors have made substantial, direct, and intellectual contribution to the work and approved it for publication. M.B. and F.S designed the study. S.G and I.G designed the experiments M.D, C.C, A.P. and A.C. developed the experiments. F.S. enrolled subjects. M.B. prepared the manuscript. M.B. and F.S revised the manuscript.

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Informed Consent Statement

The study and the data collection procedure were approved by the Ethics and Research Committee of the Città della Salute e della Scienza di Torino institutional Ethics Committee on 11/24/2014 prot. Number 116918. Informed consents were obtained verbally from the parents of the study participants and consigned in their clinical records in accordance to the Italian good clinical practices and hospital clinical investigations guidelines. The samples were anonymized before processing.

Conflicts of Interest

M.B, S.G and M.D. are foundator and scientific director, CEO and researcher c/o BioMole srl, respectively. All other authors confirm that there are no conflicts of interest.

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