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Severe preschool asthmatics have altered cytokine and anti-viral responses during exacerbation

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Abstract

Background: Preschool asthma/recurrent wheeze is a heterogeneous condition. Different clinical phenotypes have been described, including episodic viral wheeze (EVW), severe intermittent wheeze (SIW), and multiple-trigger wheeze (MTW).

Objective: To compare clinical, viral, and inflammatory/immune profiling at exacerbation between MTW, SIW, and EVW phenotypes.

Methods: Multicenter, prospective, observational cohort (VIRASTHMA-2). Children (1-5 years) with preschool asthma were enrolled during hospitalization for a severe exacerbation. History and anamnestic data, plasma, and nasal samples were collected at exacerbation (T1) and at steady state, 8 weeks later (T2), and sputum samples were collected at T1.

Results: A total of 147 children were enrolled, 37 (25%) had SIW, 18 (12.2%) EVW, and 92 (63%) MTW. They were atopic (47%), exposed to mold (22%) and cigarette smoke (50%), and prone to exacerbations (≥ 2 in the previous year in 70%). At exacerbation, at least one virus was isolated in 94% and rhinovirus in 75%, with no difference between phenotypes. Children with MTW and SIW phenotypes displayed lower plasma concentrations of IFN- γ ($P = .002$), IL-5 ($P = .020$), TNF- α ($P = .038$), IL-10 ($P = .002$), IFN- β ($P = .036$), and CXCL10 ($P = .006$) and lower levels of IFN- γ ($P = .047$) in sputum at exacerbation than children with EVW. At T2, they also displayed lower plasma levels of IFN- γ ($P = .045$) and CXCL10 ($P = .013$).

Abbreviations: AD, atopic dermatitis; AR, allergic rhinoconjunctivitis; BMI, body mass index; CSE, cigarette smoke exposure; CXCL, C-X-C motif chemokine ligand; EVW, episodic viral wheeze; FA, food allergy; GINA, Global Initiative for Asthma; ICS, inhaled corticosteroids; IFN, interferon; IL, interleukin; MTW, multiple-trigger wheeze; PRAM, Preschool Respiratory Assessment Measure; PRR, pattern recognition receptors; RSV, respiratory syncytial virus; RT-PCR, reverse transcription-polymerase chain reaction; RV, rhinovirus; SCORAD, SCORing Atopic Dermatitis severity score; SIW, severe intermittent wheeze; SPT, skin prick tests; T1, Time 1 (exacerbation); T2, Time 2 (steady state); TNF, Tumor necrosis factor; TSLP, thymic stromal lymphopoietin.

Philippe Gosset and Antoine Deschildre are senior authors and contributed equally to this work.

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Conclusion: Among preschool asthmatic children, MTW and SIW, prone to exacerbations, display lower systemic levels of Th1, Th2 cytokines, pro- and anti-inflammatory cytokines, and antiviral responses during severe virus-induced exacerbation.

KEYWORDS

asthma, infection, inflammation, phenotype, preschool children

1 | INTRODUCTION

Preschool asthma/recurrent wheeze is now recognized as a specific entity.¹ However, our understanding of its underlying mechanisms has to be improved. Despite maintenance treatment, many patients remain uncontrolled, prone to exacerbation, with a significant ongoing burden.²

Up to now, different clinical phenotypes have been described. In 2008, Brand et al³ defined the following: episodic viral wheeze (EVW: wheezing during discrete time periods, absence of symptoms between exacerbations) and multiple-trigger wheeze (MTW: wheezing during exacerbations but also symptoms between these periods). Simultaneously, in 2007, Bacharier et al⁴ identified severe intermittent wheeze (SIW), characterized by frequent and severe exacerbations, separated by periods without symptoms.

Exacerbations are one of the main clinical markers of pediatric asthma, especially in preschoolers, most often triggered by viral infections.^{2,5}

During exacerbation, a cytokine burst is occurring, characterized by the release of Th2 cytokines, alarmins, and inflammatory and antimicrobial responses.⁶ Previous studies have suggested that some asthmatic children may display a deficient antiviral response.^{2,7,8}

The VIRASTHMA-2 cohort focuses on preschool asthmatic children (<5 years), assessed at the time of a severe exacerbation and at steady state. We conducted an exploratory study with the hypothesis that children with EVW, SIW, and MTW phenotypes may display different levels of cytokines in blood and sputum at exacerbation.

After providing a description of the cohort, our primary objective was to analyze clinical, microbiological (virus identification), and

Key Message

The characterization of the VIRASTHMA-2 cohort of preschool asthmatic children highlights the burden of exacerbations in early asthma. Among them, those prone to exacerbation, multiple-trigger wheezers and severe intermittent wheezers, display lower levels of cytokines and antiviral responses during exacerbation.

immune profiling at exacerbation in EVW, MTW, and SIW patients. Our secondary objective was to conduct the same analysis at steady state.

2 | METHODS

2.1 | Study population

We initiated a multicenter, prospective, observational cohort study in eight pediatric hospital centers in the Hauts-de-France region, France, between 2015 and 2018. Preschool children (1-5 years), with a medical diagnosis of recurrent wheeze/asthma, according to the recommendations,^{1,9} were enrolled during hospitalization for a severe exacerbation (requiring a course of systemic steroids). Inclusions and sampling were performed within 48 hours following the admission and initiation of general corticosteroids.

The study was approved by ethics committee (CPP Nord-Ouest IV) and registered at ClinicalTrials.gov (NCT: 03960359). Written

informed consent was obtained from parents/caregivers. Full inclusion criteria, study design, and details on data and collection of biologic samples are described in the Appendix S1 and Table S1.

2.2 | Study design

Patients' data were collected at exacerbation (T1) and at steady state, at least 8 weeks after (T2). Blood samples to study immune profiling and virus identification were performed at both T1 and T2. Sputum samples were collected at T1.

2.3 | Definitions

2.3.1 | Phenotypes

Three groups of children were defined, according to previous recommendations: EVW: wheezing during discrete time periods (exacerbations), ≤ 1 severe exacerbation over the last 12 months, absence of symptoms between exacerbations; SIW: ≥ 2 severe exacerbations over the last 12 months, absence of interval symptoms; and MTW: wheezing during exacerbations and symptoms between these periods.^{3,4} Phenotype was defined at T2 and reviewed with the principal investigator, based on parental report of symptoms after the parents/caregivers had received education on chronic symptoms. If the child was lost to follow-up and not assessed at T2, phenotype was defined according to parental reports of symptoms at T1.

2.3.2 | History and anamnestic data

At T1, we recorded the following: history, demographics and anthropometrics, environment [cigarette smoke exposure (CSE), visible mold/moisture, pet ownership, urban living], family history, associated disorders defined according to guidelines, including atopic dermatitis (AD) (severity assessed with SCORAD: SCORing Atopic Dermatitis severity score¹⁰), allergic rhinoconjunctivitis (AR),¹¹ and food allergy (FA).¹²

2.3.3 | Asthma history and control

We assessed the following: age at first wheeze, current asthma medication, including inhaled corticosteroids (ICS) in $\mu\text{g}/\text{d}$ fluticasone or equivalent, chronic symptoms, number of severe exacerbations in the past 12 months (requiring a course ≥ 3 days of systemic steroids), day-to-day asthma control according to the Global Initiative for Asthma (GINA) criteria: well-controlled, partly controlled, uncontrolled.¹ Features of the exacerbation were noted, including Preschool Respiratory Assessment Measure (PRAM) severity score at admission in the emergency room.¹³

2.3.4 | Atopy

Full allergy diagnosis workup was performed at T2: skin prick tests (SPT) with a panel of airborne allergens and food allergens, and/or allergen-specific IgE antibodies. Atopy was diagnosed if at least one SPT result was positive (diameter ≥ 3 mm) and/or at least one specific IgE was positive (≥ 0.35 kUA/L). Serum total IgE levels (kUI/L) and peripheral blood eosinophil counts ($/\text{mm}^3$) measured at T2 were also recorded.

2.4 | Blood and sputum collection for immune profiling

Spontaneous or induced sputum (after nebulization of isotonic saline solution) were collected during a physiotherapy session.¹⁴ Plugs were isolated from the sputum, weighed, and processed. Cytospins were prepared from the cell pellets and used for cell counts. All concentrations of cytokines were measured by customized multiplex assays (Luminex®, ThermoFisher Scientific) in plasma and sputum supernatants and expressed in pg/mL. Cytokines known as being involved in the immune reaction in acute asthma and in the antiviral response were selected, based on previous relevant studies in our laboratory and in the literature.^{7,8,15} Levels of Th1 cytokines [IFN- γ], Th2 cytokines [IL-4, IL-5, IL-13], alarmins [IL-33, TSLP], Th17 cytokines [IL-17A, IL-22], pro-inflammatory cytokines [Tumor necrosis factor (TNF)- α , IL-6, C-X-C motif chemokine ligand (CXCL8)], anti-inflammatory cytokines [IL-10], antiviral response including type I interferons [IFN- β], type III interferons [IL-29], and interferon-stimulated genes [CXCL10] were assessed.

2.5 | Virus identification

Nasal swab secretions were collected at T1 and T2 using a flocked swab and specialized culture medium (Labelians, UTM1MLN™). Samples were frozen (-80°C) before RNA extraction. As previously described,⁷ nucleic acids were extracted using the Magstation System 12GC with the MagDEA® Viral DNA/RNA 200 (GC) kit (Precision System Science Co., Ltd.). Anyplex II RV16 Detection Kit (from start date to February 2016) and, thereafter, Allplex Respiratory Panels 1, 2, and 3 (Seegene) were used as multiplex reverse transcription-polymerase chain reaction (RT-PCR) assays.

2.6 | Data analysis

Categorical variables were reported as numbers (percentages) and continuous variables as median [interquartile range]. Normality was assessed graphically and using the Shapiro-Wilk test. Comparisons between the three groups (MTW, SIW, and EVW) were conducted using chi-square for categorical variables or Kruskal-Wallis test for continuous variables. Post hoc pairwise comparisons were

performed using chi-square test for categorical variables or Dunn test for continuous variables, applying the Bonferroni correction to adjust for multiple comparisons. All statistical tests were performed at the two-tailed α level of .05 using SAS software, release 9.4 (SAS Institute).

3 | RESULTS

3.1 | Study cohort

Collectively, 147 children (boys: 58.5%, median age of 30 months [23:40]) were included: 37 (25.2%) with SIW, 18 (12.2%) with EVW, and 92 (62.6%) with MTW. At T2, 135 (91.8%) children were assessed, 33 (24.4%) with SIW, 16 (11.9%) with EVW, and 86 (63.7%) with MTW (Figure 1).

Parents reported visible mold/dampness at home in 22.4%, history of mother smoke during pregnancy in 21.8%, and current CSE in 49.7% (Table 1). Children presented with early-onset wheeze, with a median age at first wheeze of 9 months (4; 25) and a median number of exacerbations in the previous year of 2 (1; 4). In all, 32.7% had a controller therapy with ICS before inclusion, with a median dose of 200 μ g/d fluticasone equivalent (200; 200). At least one associated allergic disorder was found in 57%: AD in 37.4%, with an early onset (before 4 months of age) in 19.0%; AR in 20.4%; and FA in 8.2%. Atopy was recorded in 47.1%, with sensitization to airborne allergens in 46.4% and to food allergens in 10.1%, polysensitization in 49.2% (Table 2).

Exacerbations occurred all year long, most frequent in fall (40.1% in September-November) compared with winter (17.7% in December-February), spring (20.4% in March-May), and summer (21.8% in June-August). According to the PRAM severity score at admission to the emergency room, exacerbations were moderate attacks in 55.8% and severe attacks in 12.2%. All children were prescribed a controller therapy with ICS before hospital discharge.

At least one virus was isolated in 93.8% (RV: 72.6%) (Table S2). In induced sputum ($n = 108$), median total cell number was of $13.7 \times 10^3/\text{mg}$ (5.5; 28.0) with 32.0% of macrophages (19.0; 41.5), 54.0% of neutrophils (31.0; 71.5), 3.0% of lymphocytes (1.5; 6.0), and 1.0 of eosinophils (0.5; 2.5) (Table 3). We observed detectable levels of Th1, Th2 cytokines, alarmins, Th17 cytokines, pro-inflammatory and anti-inflammatory cytokines, antiviral molecules, in both plasma ($n = 137$) and sputum ($n = 105$) at T1, with inter-individual variations (Figure S1).

At T2, 49.6% had experienced at least another exacerbation and only 58.6% had reached day-to-day asthma control. At least one virus was isolated in 66.9% (RV: 49.6%). (Table S2). We also observed detectable levels of cytokines in plasma ($n = 116$), with inter-individual variations (Figure S1).

3.2 | Comparison of clinical characteristics between MTW, SIW, and EVW

Higher rates of CSE were observed in MTW compared with SIW and EVW (57.6% vs 32.4% and 44.4%, respectively, $P = .032$), without difference in antenatal CSE (Table 1). Children with MTW and SIW were prone to exacerbation, with a median number of exacerbations in the past 12 months of 3.0 (1.0; 4.5) in MTW and 2.0 (1.0; 4.0) in SIW.

Children with MTW displayed less frequent history of early AD than SIW and EVW (10.9% vs 29.7% and 38.9%, respectively, $P = .003$). Conversely, children with MTW had more frequent history of AR than SIW and EVW (27.2% vs. 10.8% and 5.6%, respectively, $P = .028$). We did not observe any difference in the rates of FA, atopy, levels of serum total IgE, and peripheral blood eosinophil and neutrophil counts (Table 2).

At T1, clinical characteristics of exacerbations, including seasonal repartition, PRAM severity score, and length of oxygen need, were comparable within MTW, SIW, and EVW (not shown).

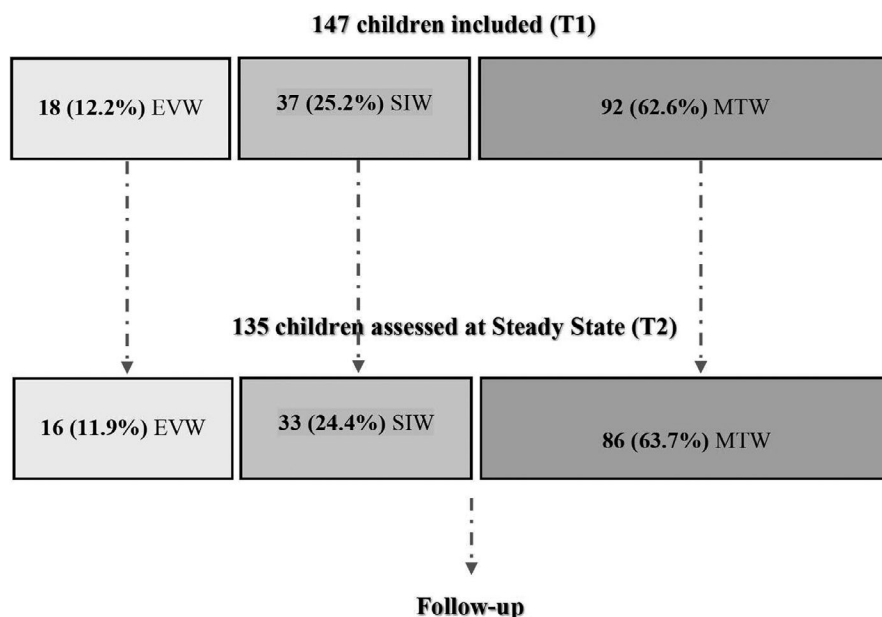


FIGURE 1 Flowchart of the population. EVW, episodic viral wheeze; MTW, multiple-trigger wheeze; SIW, severe intermittent wheeze; T1, Time 1 (at exacerbation); T2, Time 2 (at steady state)

TABLE 1 Demographic and environmental data

	Total population n = 147	MTW n = 92	SIW n = 37	EVW n = 18	Global P-value
Demographics and anthropometrics					
Median age (in mo)	30.0 (23.0; 40.0)	30.5 (24.5; 38.0)	29.0 (21.0; 42.0)	28.5 (26.0; 38.0)	.92
Male	86 (58.5)	53 (57.6)	21 (56.8)	12 (66.7)	.75
Z score for BMI	-0.8 (-1.3; -0.1)	-0.8 (-1.4; -0.1)	-0.8 (-1.2; 0.1)	-0.6 (-1.0; -0.2)	.63
Personal history					
Birthweight (in g)	3300 (3020; 3650)	3200 (2980; 3590)	3430 (3130; 3700)	3453 (3200; 3780)	.083
Mother smoke during pregnancy	32 (21.8)	21 (22.8)	7 (18.9)	4 (22.2)	.89
Breastfeeding	88 (59.9)	54 (58.7)	23 (62.2)	11 (61.1)	.93
Environment					
Inner-city	119 (81.0)	73 (79.3)	30 (81.1)	16 (88.9)	.64
Visible mold/dampness at home	33 (22.4)	23 (25.0)	6 (16.2)	4 (22.2)	.56
Dog and/or cat ownership	61 (41.5)	40 (43.5)	18 (48.6)	3 (16.7)	.064
Cigarette smoke exposure	73 (49.7)	53 (57.6) ^a	12 (32.4) ^a	8 (44.4)	.032
Sibling in childcare facility	55 (37.9)	33 (36.7)	15 (40.5)	7 (38.9)	.92
Participant in childcare facility	77 (52.7)	50 (54.9)	19 (51.4)	8 (44.4)	.70
Parental employment and education					
Father employed	127 (87.6) (n' = 145)	75 (83.3) (n' = 90)	34 (91.9)	18 (100.0)	.10
Mother employed	94 (64.4) (n' = 146)	56 (60.9) (n' = 92)	27 (75.0) (n' = 36)	11 (61.1)	.31
Father completed higher education	65 (52.4) (n' = 124)	34 (45.3) (n' = 75)	21 (67.7) (n' = 31)	10 (55.6)	.11
Mother completed higher education	80 (61.1) (n' = 131)	45 (55.6) (n' = 81)	26 (76.5) (n' = 34)	9 (56.3) (n' = 16)	.10

Note: P-values < .05 are indicated in bold letters.

Values are reported as frequency (percentage) or median (interquartile range). Comparisons between the three groups (MTW, SIW, and EVW) were conducted using chi-square test for categorical variables or Kruskal-Wallis test for continuous variables.

Abbreviations: BMI, body mass index; MTW, multiple-trigger wheeze; SIW, severe intermittent wheeze.

^aIdentifies the significant pairwise post hoc comparisons after Bonferroni's correction.

3.3 | Biologic characteristics at exacerbation and steady state according to phenotypes

There was no significant difference in the identification of viruses between phenotypes (Table S2). Sputum characteristics were similar, with the exception of higher levels of epithelial cells in sputum from MTW compared with SIW and EVW [4.5% (1.5; 12.0) vs 2.0 (1.0; 6.0) and 3.5 (2.0; 10.0), respectively, $P = .042$] (Table 3).

Compared with EVW, children with MTW and SIW displayed significantly lower plasma cytokine concentrations of IFN- γ ($P = .002$) and IL-5 ($P = .020$) and lower levels of IFN- γ ($P = .047$) in sputum (Table 4), but no difference in IL-4, IL-13, and alarmins plasma and sputum concentrations. They also presented with lower blood concentrations of TNF- α ($P = .038$), IL-10 ($P = .002$), IFN- β ($P = .036$), and CXCL10 ($P = .006$). There was no difference regarding levels of Th17 cytokines.

At T2, there was no difference in virus identification between the three groups (Table S2). Regarding immune profiling, MTW and SIW displayed significantly lower plasma levels of IFN- γ than EVW ($P = .045$) and MTW displayed lower plasma levels of CXCL10 than the two others ($P = .013$) (Table 5). Levels of Th2 cytokines, alarmins, Th17 cytokines, pro- and anti-inflammatory cytokines, and type I and III IFNs were comparable.

4 | DISCUSSION

The VIRASTHMA-2 cohort enrolled a large population of pre-school asthmatic children at the time of a severe exacerbation. Their characteristics highlight the early burden of asthma, especially the unmet needs in maintenance treatment and prevention

TABLE 2 Asthma history and associated allergic disorders

	Total population n = 147	MTW n = 92	SIW n = 37	EVW n = 18	Global P-value
Family atopy status					
Atopy in one or 2 parents or sibling	109 (74.1)	69 (75.0)	27 (73.0)	13 (72.2)	.95
Asthma in one or 2 parents or sibling	78 (53.1)	51 (55.4)	18 (48.6)	9 (50.0)	.75
Asthma history					
Age at first wheeze (in mo)	9.0 (4.0; 25.0)	9.0 (4.5; 23.5)	10.0 (6.0; 27.0)	8.0 (4.0; 25.0)	.51
Number of exacerbations in the previous 12 mo	2.0 (1.0; 4.0)	3.0 (1.0; 4.5) ^b	2.0 (1.0; 4.0) ^c	0.0 (0.0; 0.0) ^{b,c}	<.001
≥2 courses of oral steroids in the previous 12 mo	103 (70.1)	66 (71.7) ^{a,b}	37 (100.0) ^{a,c}	0 (0.0) ^{b,c}	<.001
Asthma day-to-day control before inclusion (GINA)					
Well-controlled	75 (51.0)	20 (21.7)	37 (100.0)	18 (100.0)	NA
Partly controlled	45 (30.6)	45 (48.9)	0 (0.0)	0 (0.0)	
Uncontrolled	27 (18.4)	27 (29.3)	0 (0.0)	0 (0.0)	
Controller therapy with ICS before inclusion	48 (32.7)	35 (38.0)	11 (29.7)	2 (11.1)	.076
Doses of ICS (μg/d fluticasone)	200 (200.0; 200.0)	200 (200.0; 200.0)	200 (200.0; 200.0)	725.0 (200.0; 1250)	NA
Associated allergic disorders					
At least one associated allergic disorder	83 (56.5)	53 (57.6)	18 (48.6)	12 (66.7)	.42
Atopic dermatitis	55 (37.4)	28 (30.4)	17 (45.9)	10 (55.6)	.061
Early atopic dermatitis (<4 mo of age)	28 (19.0)	10 (10.9) ^{a,b}	11 (29.7) ^a	7 (38.9) ^b	.003
SCORAD score if current lesions	14.0 (7.8; 23.3) (n' = 30)	21.5 (9.5; 29.0) (n' = 14)	11.0 (7.1; 16.4) (n' = 11)	15.0 (7.8; 16.0) (n' = 5)	NA
Allergic rhinoconjunctivitis	30 (20.4)	25 (27.2)	4 (10.8)	1 (5.6)	.028
Food allergy	12 (8.2)	6 (6.5)	3 (8.1)	3 (16.7)	.26
Atopy ^{†,a}	65 (47.1) (n' = 138)	39 (44.3) (n' = 88)	15 (44.1) (n' = 34)	11 (68.8) (n' = 16)	.18
Sensitization to airborne allergens	64 (46.4) (n' = 138)	39 (44.3) (n' = 88)	15 (44.1) (n' = 34)	10 (62.5) (n' = 16)	.39
Sensitization to food allergens	14 (10.1) (n' = 138)	8 (9.1) (n' = 88)	3 (8.8) (n' = 34)	3 (18.8) (n' = 16)	.53
Polysensitization	32 (23.2) (n' = 138)	18 (20.5) (n' = 88)	8 (23.5) (n' = 34)	6 (37.5) (n' = 16)	.33
Serum total IgE levels (kuia/L)	54.0 (13.9; 179.0) (n' = 125)	64.2 (14.2; 172.0) (n' = 78)	40.2 (13.5; 179.0) (n' = 31)	68.5 (14.1; 319.5) (n' = 16)	.63
Peripheral blood eosinophil counts (T2) (cells/mm ³)	400 (200; 800) (n' = 99)	400 (100; 800) (n' = 60)	300 (200; 900) (n' = 25)	300.0 (100.0; 700.0) (n' = 14)	.96
Peripheral blood neutrophil counts (T2) (cells/mm ³)	2950 (2300; 3900) (n' = 99)	3000 (2200; 4400) (n' = 60)	2700 (2300; 3800) (n' = 25)	3300 (2500; 4300) (n' = 14)	.88

Note: P-values < .05 are indicated in bold letters.

Values are reported as frequency (percentage) or median (interquartile range). Comparisons between the three groups (MTW, SIW, and EVW) were conducted using chi-square test for categorical variables or Kruskal-Wallis test for continuous variables. a, b, c identify the significant pairwise post hoc comparisons after Bonferroni's correction.

Abbreviations: GINA, Global Initiative for Asthma; ICS, inhaled corticosteroids; MTW, multiple-trigger wheeze; NA, not available (n < 8); SCORAD, SCORing Atopic Dermatitis severity score; SIW, severe intermittent wheeze.

[†]Atopy: at least one positive skin prick test (diameter ≥3 mm) and/or specific IgE antibodies (≥0.35 kuia/L).

of exacerbations.^{4,5,16,17} They were frequently atopic, exposed to mold and cigarette smoke, and prone to exacerbations. At inclusion, at least one virus was isolated in 94% and RV in 75%,

with no difference between phenotypes. Children with MTW and SIW phenotypes displayed lower plasma concentrations of IFN-γ, IL-5, TNF-α, IL-10, IFN-β, and CXCL10 and lower levels of IFN-γ in

TABLE 3 Analysis of cells in sputum at exacerbation (T1)

Sputum	Total population n = 147	MTW n = 92	SIW n = 37	EVW n = 18	Global P-value
Weight (mg)	195.0 (100.0; 350.0) (n' = 111)	190.0 (100.0; 360.0) (n' = 67)	200.0 (105.0; 425.0) (n' = 28)	175.0 (95.5; 200.0) (n' = 16)	.48
Cells (n × 10 ³ /mg)	13 730 (5509; 28 043) (n' = 108)	12 174 (5421; 28 000) (n' = 65)	16 000 (7000; 30 000) (n' = 27)	14 688 (5409; 24 566) (n' = 16)	.64
% Macrophages	32.0 (19.0; 41.5) (n' = 103)	33.0 (21.0; 45.0) (n' = 61)	30.0 (17.0; 41.0) (n' = 27)	24.0 (18.0; 34.0) (n' = 15)	.28
% Neutrophils	54.0 (31.0; 71.5) (n' = 103)	49.5 (26.0; 66.5) (n' = 61)	55.0 (44.0; 72.0) (n' = 27)	65.0 (37.5; 75.0) (n' = 15)	.12
% Lymphocytes	3.0 (1.5; 6.0) (n' = 103)	3.0 (2.0; 6.0) (n' = 61)	2.5 (1.5; 5.0) (n' = 27)	3.5 (2.5; 5.0) (n' = 15)	.44
% Eosinophils	0.5 (0.0; 1.5) (n' = 103)	0.5 (0.0; 1.5) (n' = 61)	1.0 (0.0; 2.5) (n' = 27)	0.5 (0.5; 1.5) (n' = 15)	.088
Basophils >0%, n (%)	3 (2.9) ^{†,a} (n' = 103)	2 (3.2) (n' = 61)	1 (3.7) (n' = 27)	0 (n' = 15)	NA
% Epithelial cells	3.5 (1.0; 10.0) (n' = 101)	4.5 (1.5; 12.0) (n' = 59)	2.0 (1.0; 6.0) (n' = 27)	3.5 (2.0; 10.0) (n' = 15)	.042

Note: P-values < .05 are indicated in bold letters.

Values are reported as median (interquartile range) excepted otherwise indicated. Comparisons between the three groups (MTW, SIW, and EVW) were conducted using Kruskal-Wallis test.

Abbreviations: MTW, multiple-trigger wheeze; NA, not applicable (n < 8); SIW, severe intermittent wheeze.

^aOnly three positive values were found: 0.5 (MTW), 0.5 (SIW), and 1.0 (MTW).

sputum at exacerbation than children with EVW. At T2, they also displayed lower plasma levels of IFN- γ and for MTW, CXCL10.

In previous studies, early and multiple allergic sensitizations have not only been associated with severity but also with persistent asthma and long-term decline of lung function.^{18,19} The impact of the environment has also been shown in numerous studies, especially for CSE,²⁰ molds,²¹ and pollution.²²

We observed higher rates of viral infections during exacerbations compared to relevant studies in older children and adults.²³

Rhinoviruses were confirmed as the main triggers.^{8,15} Viruses were also detected at steady state, as previously reported in both healthy and asthmatic children, with a higher frequency in the latter.^{23,24}

Our primary objective was to assess the differences between MTW, SIW, and EVW phenotypes. They were similar in terms of most clinical and microbiological features, except for atopic comorbidities. Atopic dermatitis was more frequent in EVW and SIW children whereas rhinitis was more frequent in MTW, without differences in sensitizations. This could be related to a particular sensitivity of the skin or the respiratory mucosa. The high levels of epithelial cells in sputum from MTW may reflect epithelial damages in response to viruses and/or inflammation.²⁵ Additional data are needed in order to assess interactions between epithelial barriers, allergic diseases, and asthma development. Airway, skin, and intestinal microbiota may also be key players, as recently reported in a study conducted in preschool wheezers.²⁶

Regarding immune profiling, we observed that MTW and SIW displayed lower plasma concentrations of IFN- γ , IL-5, TNF- α , IL-10, IFN- β , and CXCL10 and lower sputum levels of IFN- γ than EVW. These data suggest an overall defective response to viral infections in children with MTW and SIW, who are those prone to frequent exacerbation, with lower production of IFN- β and IFN-stimulated

gene CXCL10, in response to viruses. Hence, levels of other cellular and soluble factors, such as IFN- γ , IL-5, TNF- α , were also lower in the most severe patients (MTW and SIW), compared to the EVW group. We cannot exclude that this reduced inflammatory response in the most severe patients may be influenced by concomitant treatment with ICS, although we did not observe a significant difference regarding treatment between phenotypes. We also observed lower circulating levels of IL-10, which could reflect an associated impairment of regulatory mechanisms. Previous studies, conducted in school-aged children and adults, have shown conflicting results regarding the response to virus infection in asthmatic patients.^{2,8,27,28} In a study conducted in 72 school-aged children with allergic asthma, we have also reported an impaired antiviral response during severe exacerbation in a subgroup of children prone to viral carriage at steady state and displaying modifications of PRRs.⁷ Although we did not observe any difference in viruses at steady state in the present study, we observed lower circulating levels of IFN- γ in MTW and SIW and lower levels of CXCL10 in MTW, which might reflect an underlying defect of virus sensors' expression and/or function at baseline. Other studies have suggested differential baseline levels of airway cytokines, including IL-4, IL-13, and CXCL10 in patients with severe asthma compared with non-severe asthma.²⁹ Children prone to exacerbation despite ICS maintenance treatment constitute a distinct phenotype that may be associated with altered inflammatory and antiviral response, alongside allergic sensitization and underlying airway inflammation.³⁰ New treatment strategies targeting this distinct phenotype are urgently needed.

There are some limitations to our study. We selected a population of children on the occurrence of a severe exacerbation, leading to hospitalization, thus not representative of all preschool children with recurrent wheeze/asthma. We defined clinical phenotypes: EVW, MTW, and SIW, which have been questioned in

TABLE 4 Inflammatory and immune profile in blood and sputum at exacerbation (T1)

	Total population n = 147	MTW n = 92	SIW n = 37	EVW n = 18	Global P-value
Plasma	n' = 137	n' = 88	n' = 33	n' = 16	
IFN- γ	1.3 (0.0; 47.0)	0.0 (0.0; 38.9) ^a	1.3 (0.0; 37.5)	52.5 (12.2; 169.5) ^a	.002
IL-4	7.5 (4.9; 17.7)	6.4 (4.3; 12.2)	7.4 (5.8; 14.0)	15.1 (8.8; 29.4)	.060
IL-5	7.7 (0.0; 26.6)	4.9 (0.0; 20.2)	10.8 (0.9; 21.7)	26.2 (9.4; 37.1)	.020
IL-13	4.7 (1.3; 9.1)	4.5 (0.1; 8.7)	4.7 (1.5; 9.0)	6.6 (3.9; 13.7)	.084
IL-33	1.9 (0.0; 22.9)	1.3 (0.0; 21.4)	3.5 (0.0; 13.5)	9.0 (0.3; 56.7)	.21
TSLP	14.1 (6.0; 29.9)	13.9 (6.0; 34.0)	15.1 (8.9; 25.6)	14.6 (1.0; 40.9)	.88
IL-17A	3.7 (1.0; 15.2)	2.9 (0.2; 12.9)	4.0 (1.2; 14.1)	11.1 (2.2; 23.4)	.090
IL-22	25.1 (9.3; 75.8)	24.5 (4.6; 62.0)	20.1 (8.9; 75.8)	37.0 (16.8; 104.7)	.41
TNF- α	7.5 (3.3; 15.2)	6.7 (3.0; 13.7)	9.0 (3.3; 17.4)	13.3 (8.5; 19.6)	.038
IL-6	7.7 (0.0; 19.5)	5.3 (0.0; 19.5)	9.3 (0.0; 16.5)	14.7 (6.3; 29.4)	.087
CXCL8	5.7 (1.5; 11.5)	5.3 (0.2; 10.4)	5.9 (3.9; 10.3)	8.3 (3.8; 18.8)	.16
IL-10	0.0 (0.0; 1.0)	0.0 (0.0; 0.9) ^a	0.0 (0.0; 0.4) ^b	1.3 (0.2; 4.1) ^{a,b}	.002
IFN- β	(0.0; 2.7)	0.0 (0.0; 0.5)	0.0 (0.0; 2.9)	2.6 (0.0; 5.3)	.036
IL-29	136.0 (72.4; 269.2)	136.0 (71.0; 211.7)	136.0 (71.4; 282.7)	189.4 (78.0; 332.4)	.47
CXCL10	71.1 (11.9; 230.0)	21.8 (10.2; 194.0) ^a	89.5 (9.5; 259.6)	202.8 (123.7; 355.5) ^a	.006
Sputum	n' = 105	n = 65	n = 26	n = 14	
IFN- γ	7.7 (0.6; 31.0)	3.0 (0.0; 28.0)	10.2 (1.3; 24.9)	21.6 (12.2; 37.5)	.047
IL-4	7.5 (3.7; 30.2)	2.5 (0.0; 8.7)	3.2 (1.1; 8.3)	3.5 (2.2; 8.3)	.72
IL-5	11.4 (4.4; 21.9)	11.4 (3.7; 21.8)	15.6 (7.4; 26.6)	10.8 (5.3; 23.9)	.60
IL-13	4.1 (2.8; 6.9)	3.4 (1.4; 5.7)	4.6 (3.5; 7.9)	3.9 (3.3; 6.2)	.12
IL-33	4.2 (0.0; 17.6)	4.3 (0.0; 17.6)	4.1 (0.0; 17.6)	0.7 (0.0; 17.7)	.99
TSLP	11.6 (6.1; 18.1)	12.0 (6.6; 18.1)	9.0 (6.0; 20.4)	6.1 (4.3; 15.6)	.35
IL-17A	2.7 (0.2; 8.3)	5.4 (3.3; 32.8)	8.1 (5.1; 18.4)	13.8 (8.8; 26.9)	.27
IL-22	69.3 (38.3; 137.4)	64.2 (36.9; 137.7)	88.0 (38.3; 176.7)	65.6 (42.1; 99.8)	.79
TNF- α	13.3 (7.2; 24.8)	13.7 (6.1; 25.0)	13.5 (7.2; 30.5)	10.8 (7.2; 18.9)	.61
IL-6	92.8 (34.7; 214.2)	104.6 (38.8; 227.8)	98.6 (24.7; 321.1)	41.3 (15.8; 164.8)	.41
CXCL8	525.1 (395.3; 729.0)	515.7 (414.3; 680.0)	601.3 (402.4; 776.8)	515.9 (325.9; 1029)	.85
IL-10	(0.0; 0.8)	0.0 (0.0; 0.5)	0.3 (0.0; 1.1)	0.2 (0.0; 0.8)	.34
IFN- β	0.0 (0.0; 4.5)	0.0 (0.0; 1.4)	0.0 (0.0; 5.7)	3.5 (0.0; 5.2)	.061
IL-29	95.7 (46.7; 174.9)	95.7 (44.9; 179.9)	91.6 (46.7; 236.2)	102.8 (72.4; 136.0)	.81
CXCL10	820.0 (293.7; 2657)	816.4 (293.7; 2312)	627.0 (188.7; 2789)	1815 (722.2; 2810)	.25

Note: P-values < .05 are indicated in bold letters.

All concentrations were measured by multiplex assays (Luminex®, ThermoFisher Scientific) and expressed in pg/mL. Values are reported as median (interquartile range). Comparisons between the three groups (MTW, SIW, and EVW) were conducted using Kruskal-Wallis test. a, b identify the significant pairwise post hoc comparisons after Bonferroni's correction.

Abbreviations: CXCL, C-X-C motif chemokine ligand; IFN, interferon; IL, interleukin; MTW, multiple-trigger wheeze; SIW, severe intermittent wheeze; TSLP, thymic stromal lymphopoietin.

the past years,³¹ although these definitions remained in use in recent studies on preschool asthma.²⁶ Persistence of phenotypes over time is another issue that we will address with data obtained after long-term follow-up and the analysis of the clinical and functional trajectories in the upcoming years. Due to its design as a study conducted in real-life practice, we did not include healthy controls. However, we included EVW patients with a milder phenotype. Another bias could be related to the sampling in some

children with pre-existing maintenance treatment and after initiation of systemic corticosteroids. All the samples were collected within 48 hours from the start of systemic corticosteroids and the enrolled children received the same treatment modalities at exacerbation. Finally, due to its exploratory design, no formal sample size calculation was conducted. Therefore, we cannot exclude that some differences between the phenotypes could be overlooked due to a lack of adequate statistical power. Although we included

TABLE 5 Inflammatory and immune profile in blood at steady state (T2)

	Total population n = 147	MTW n = 92	SIW n = 37	EVW n = 18	Global P-value
Plasma	n' = 116	n' = 72	n' = 31	n' = 13	
IFN- γ	11.4 (0.0; 35.1)	0.2 (0.0; 31.8)	11.6 (0.0; 34.3)	34.3 (11.6; 176.5)	.045
IL-4	8.5 (4.5; 12.6)	8.2 (4.9; 12.6)	7.9 (2.3; 11.4)	8.8 (3.7; 52.6)	.40
IL-5	9.1 (1.8; 20.6)	10.8 (2.6; 18.1)	7.4 (1.4; 19.3)	19.0 (5.1; 42.1)	.35
IL-13	4.1 (1.3; 6.9)	3.7 (0.7; 6.6)	4.1 (1.7; 6.9)	5.8 (3.6; 15.2)	.14
IL-33	2.5 (0.0; 22.9)	2.2 (0.0; 18.7)	2.5 (0.0; 22.9)	7.7 (0.0; 43.2)	.48
TSLP	15.5 (6.8; 31.7)	17.9 (5.6; 28.6)	15.1 (7.7; 35.4)	15.7 (7.7; 47.8)	.83
IL-17A	4.0 (1.5; 14.1)	3.9 (1.1; 12.8)	4.8 (2.2; 11.8)	10.7 (1.9; 23.6)	.41
IL-22	25.2 (9.3; 86.1)	25.2 (8.2; 98.0)	23.7 (9.7; 62.5)	45.8 (17.1; 64.1)	.81
TNF- α	8.1 (3.7; 15.3)	8.1 (3.3; 16.0)	7.2 (4.3; 12.9)	11.1 (7.4; 15.7)	.37
IL-6	7.5 (0.0; 14.0)	7.3 (0.0; 13.6)	7.3 (0.0; 11.8)	14.0 (2.6; 25.2)	.23
CXCL8	3.8 (1.2; 8.7)	3.81 (0.02; 9.36)	3.57 (1.11; 5.72)	5.33 (2.43; 13.05)	.51
IL-10	0.0 (0.0; 0.7)	0.0 (0.0; 0.6)	0.0 (0.0; 0.5)	0.0 (0.0; 2.5)	.45
IFN- β	0.0 (0.0; 2.9)	0.0 (0.0; 2.4)	0.0 (0.0; 2.9)	0.0 (0.0; 4.7)	.34
IL-29	116.7 (64.7; 296.4)	126.3 (64.7; 305.5)	102.0 (54.1; 184.9)	170.8 (74.7; 341.6)	.64
CXCL10	83.0 (10.1; 202)	14.5 (7.5; 182.7)	140.5 (12.8; 259.6)	200.1 (90.8; 266.2)	.013

Note: P-values < .05 are indicated in bold letters.

All concentrations were measured by multiplex assays (Luminex[®], ThermoFisher Scientific) and expressed in pg/mL. Values are reported as median (interquartile range). Comparisons between the three groups (MTW, SIW, and EVW) were conducted using Kruskal-Wallis test.

Abbreviations: CXCL, C-X-C motif chemokine ligand; IFN, interferon; IL, interleukin; MTW, multiple-trigger wheeze; SIW, severe intermittent wheeze; TSLP, thymic stromal lymphopoietin.

a limited number of patients compared with studies conducted in adults and older children, it remains one of the most important cohort of preschool children assessed during an acute exacerbation and at steady state, with limited number of lost to follow-up and missing data. Another strength of our study is its prospective and multicenter design. We also confirmed that sputum collection is feasible in preschool children.¹⁴

In conclusion, our study not only highlights the burden of asthma in preschoolers, but also shows that severe cases, who are prone to exacerbation, may display impaired inflammatory and antiviral response at exacerbation. Further studies assessing preschool

wheeze/asthma heterogeneity through various endotypes are urgently needed in order to anticipate the future morbidity risk and hence develop new treatment strategies.

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CONFLICT OF INTEREST

All authors have no conflict of interest regarding this study.

AUTHOR CONTRIBUTION

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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Severe preschool asthmatics have altered cytokine and anti-viral responses during exacerbation – Supporting information

Appendix S1

1. Study population

We initiated a multicenter, prospective, observational cohort study in eight pediatric hospital centers in the Hauts-de-France region, France, between 2015 and 2018. We enrolled preschool children aged 1 to 5 years, with a medical diagnosis of asthma, according to the recommendations, i.e. with at least 3 episodes of wheezing since birth or with symptoms of asthma between episodes (1,9), during hospitalization for a severe exacerbation (requiring a course of general steroids). Inclusions and sampling were performed within 48 hours after admission and initiation of general corticosteroids. Non-inclusion criteria were: children with a history of chronic disease other than asthma, a history of preterm birth (< 36 weeks of amenorrhea).

The study was approved by the ethics committee (CPP Nord-Ouest IV) and registered at ClinicalTrial.gov (NCT: 03960359). Written informed consent was obtained from parents/caregivers.

Study design

The study design of the VIRASTHMA-2 study is shown in Suppl Table 1. Patients' data were collected at exacerbation (T1) and at steady state at least 8 weeks after the exacerbation (T2). Blood samples to study immune profiling and virus identification were performed at both T1 and T2. Sputum samples were only collected at T1 to assess immune profiling and bacterial identification. Patients will then be followed up until the age of 7. A sub-population of patients in Lille University Hospital Center will be followed-up until the age of 17, with an intermediate assessment at 12.

2. Definitions

3.1. Phenotypes

Three groups of children were defined, according to previous recommendations: Episodic-Viral Wheeze (EVW): wheezing during discrete time periods (exacerbations), ≤ 1 severe exacerbation over the last 12 months, absence of symptoms between exacerbations; Severe Intermittent Wheeze (SIW): ≥ 2 severe exacerbations over the last 12 months; and Multiple-trigger wheeze (MTW): wheezing during exacerbations but also symptoms (cough, exercise-induced symptoms,...) between these periods (3,4). Phenotype was defined after 2 months of follow-up, at T2 and reviewed with the principal investigator of the study, based on parental report of symptoms after the parents / caregivers had received education on chronic symptoms. Phenotype was then applied retrospectively at T1. If the child was lost to follow-up and not assessed at T2, phenotype was defined according to parental reports of symptoms at T1.

3.2. History and anamnestic data

At T1, history and anamnestic data included: demographics and anthropometrics, environment, family history, associated disorders, features of the exacerbation.

- Anthropometry:

Body mass index (BMI) Z-score were generated using French growth data by Rolland-Cachera.

- Environment:

Questionnaires were performed to assess inborn and outborn exposure, based on the declaration of the parents: cigarette smoke exposure (CSE), visible mold/moisture exposure, pet ownership, urban living.

- Associated atopic disorders:

History of atopic dermatitis (AD) was noted. In case of current AD, its severity was assessed with SCORAD: Scoring Atopic Dermatitis severity score (10). Allergic rhinoconjunctivitis (AR) was retained in case of one or more chronic symptoms including sneezing, itching, nasal congestion and rhinorrhea (11). Food allergy (FA) was retained if a patient presented with history of clinical manifestations of immediate hypersensitivity (skin, respiratory, circulatory or digestive) following ingestion of food(12).

3.3. Asthma history and control

Past history of asthma exacerbations and age at first wheeze were recorded. We assessed: current asthma medication, including inhaled corticosteroids (ICS) in $\mu\text{g/day}$ fluticasone or equivalent, presence of chronic symptoms, number of severe exacerbations in the past 12 months (requiring a course ≥ 3 days of systemic steroids), day-to-day asthma control based on symptoms (breath, cough, breathlessness, impact on activity and social behavior) and classified according to the GINA (Global Initiative for Asthma) criteria: well-controlled, partly controlled, uncontrolled (1). Features of the exacerbation were noted, including severity, assessed using the PRAM (Preschool Respiratory Assessment Measure) score at admission in the emergency room (before treatment), to distinguish mild asthma (score of 0-3), moderate asthma (score of 4-7) or severe asthma (score of 8-12) (13). Length of stay and oxygen need were also recorded (in days).

3.4. Atopy

For each patient, full allergy diagnosis work-up was performed at T2: skin prick tests (SPT) with a panel of airborne allergens (house dust mite, cat and dog dander, birch and grass pollens, *Alternaria*, other allergen depending on the environment, ALK Abello) and food allergens (cow's milk, egg, other allergens in cases of suspicion of FA, ALK Abello), allergen

specific IgE antibodies in blood. Atopy was diagnosed if at least one SPT result was positive (diameter ≥ 3 mm) and/or at least one specific IgE was positive (≥ 0.35 kUA/l). Serum total IgE levels (kUI/l), peripheral blood eosinophil counts (cells/mm³), measured at T2, were also recorded. In a sub-population of patients (in Lille University Hospital center) ImmunoCAP™ ISAC (ThermoFischer Scientific) were also performed (data not shown).

3. Blood and sputum collection for immune profiling

Sputum and blood samples were collected within the first 2 days of exacerbation. Spontaneous or induced sputum samples (after nebulization of isotonic saline solution) were collected during a physiotherapy session. Biological samples were quickly routed to the laboratory and processed within 4 hours. After processing (as follows), all resulting samples (plasma, RNAs, cultures supernatants) were stored and frozen at -80°C for later use.

Plasma processing:

Plasma from blood samples were used to measure cytokine concentrations. Peripheral Blood mononuclear cells (PBMC) were also extracted and isolated using a Ficoll-Paque density gradient (7). After washings and resuspension, cells were used for cell cultures (24 hours), immediate phenotypic characterization by flow cytometry and RNA extractions (data not shown).

Sputum processing:

Spontaneous or induced sputum samples (after nebulization of isotonic saline serum) were collected during a physiotherapy session (14). Plugs were isolated from the sputum, weighted and processed. They were diluted with sputolysin (VWR) and then, sputum fluids and cells were separated by centrifugation. The cell pellets were used for differential cell counts analyzed on cytopins, phenotypic characterization by flow cytometry and RNA extractions (data not shown) and the supernatants for cytokine measurements.

Cytokine measurements:

All concentrations were measured by customized multiplex assays (Luminex®, ThermoFisher Scientific) in plasma and supernatants and expressed in pg/ml. Cytokines known as being involved in the immune reaction in acute asthma and in the anti-viral response were selected, based on previous relevant studies in our laboratory and in the literature (7,8,15). Levels of Th1 cytokines [IFN- γ], Th2 cytokines [IL-4, IL-5, IL-13], alarmins [IL-33, TSLP], Th17 cytokines [IL-17, IL-22], pro-inflammatory cytokines [IL-1 β , Tumor necrosis factor (TNF)- α , IL-6, C-X-C motif chemokine ligand (CXCL8)], anti-inflammatory cytokines [IL-10], anti-viral response including type I interferons [IFN- β], type III interferons [IL-29], and Interferon-stimulated genes [CXCL10], chemokines (CCL5, CCL20) in plasma, sputum fluids and supernatants from cultures of PBMC were assessed. Levels of IL-1 β , CCL5, CCL20 in plasma and sputum fluids as well as levels of all cytokines in supernatants from cultures of PBMC are not shown.

4.2 Virus identification

Nasal swab secretions were performed for each patient at T1 and at T2 using a flocked swab and specialized culture medium (Labelians, UTM1MLN™). Samples were frozen (–80 °C) before RNA extraction. As previously described, nucleic acids were extracted from 200 μ L of each specimen using the Magstration System 12GC with the MagDEA® Viral DNA/RNA 200 (GC) kit (Precision System Science Co., Ltd.; Japan) (7). Commercially available multiplex reverse transcription–polymerase chain reaction (RT-PCR) assays were used to screen for a panel of respiratory viral pathogens including influenza virus A and B, respiratory syncytial virus (RSV) A and B, human adenovirus (HAdV), human metapneumovirus, coronaviruses 229E, NL63 and OC43, parainfluenzavirus 1–4, rhinovirus (RV) A/B/C, human enterovirus, and bocavirus 1–4. From the beginning of the study to

February 8th, 2016 the Anyplex II RV16 Detection Kit (Seegene, Seoul, Korea) was used. Thereafter, Allplex Respiratory Panel 1, 2 and 3 (Seegene, Seoul, Korea) was used.

4. Data analysis

Categorical variables were reported as numbers (percentages) and continuous variables as median [interquartile range (IQR)]. Normality was assessed graphically and using the Shapiro-Wilk test. Comparisons between the three groups (MTW, SIW and NSIW) were conducted using Chi-square for categorical variables or Kruskal-Wallis test for continuous variables. Post-hoc pairwise comparisons were performed using the Chi-square test for categorical variables or the Dunn test for continuous variables, applying the Bonferroni correction to adjust for multiple comparisons. All statistical tests were performed at the two-tailed α level of 0.05 using SAS software, release 9.4 (SAS Institute, Cary, NC).

Supplemental Figure 1:

Levels of Th1 cytokines, Th2 cytokines and alarmins, Th17 cytokines, pro- and anti-inflammatory cytokines, anti-viral response in plasma (upper panel, n=137) and sputum fluids (lower panel, n=105) at exacerbation.

IFN: Interferon; IL: Interleukine; TNF- α ; Tumor necrosis factor alpha; Thymic stromal lymphopietin; CXCL: C-X-C motif chemokine ligand; Pro / anti inf: pro and anti-inflammatory cytokines.

All concentrations were measured by multiplex assays (Luminex®, ThermoFisher Scientific) and expressed in pg/ml

Supp TABLE 1: Study design – VIRASTHMA-2

Exacerbation (T1): Hospitalisation	8 weeks later (T2): Steady state
- Past history - Environmental exposure - Asthma history, medication and control (GINA†) - Anthropometry - Clinical features of exacerbation - Blood sample: > Immune profiling (levels of cytokines in plasma, preparation of PBMCs‡ for flow cytometry, culture, RNA) - Induced sputum: > Immune profiling (levels of cytokines in supernatants, preparation of cells for flow cytometry, RNA) > Bacterial identification (culture) - Nasal swab sample: > Virus identification (RT-PCR§)	- Recent medical history - Asthma medication and control (GINA) - Anthropometry - Definition of phenotype : > Episodic-Viral Wheeze (EVW¶) > Severe Intermittent Wheeze (SIW††) > Multiple-Trigger Wheeze (MTW‡‡) - Full allergy diagnosis work-up: > SPT§§ with a panel of airborne and food allergens > Allergen specific IgE antibodies in blood > Serum total IgE level (kUI/l) > Peripheral blood eosinophil counts (cells/mm³) > ImmunoCAP™ ISAC (<i>sub-population</i>) - Blood sample: > Immune profiling (levels of cytokines in plasma, preparation of PBMCs for flow cytometry, culture, RNA) - Nasal swab sample: > Virus identification (RT-PCR§)

†GINA: global initiative for asthma; ‡PBMCs: peripheral blood mononuclear cells; §RT-PCR: reverse transcription polymerase chain reaction; ¶EVW: episodic viral wheeze; ††SIW: severe intermittent wheeze; ‡‡MTW: multiple-trigger wheeze; §§SPT: skin prick tests

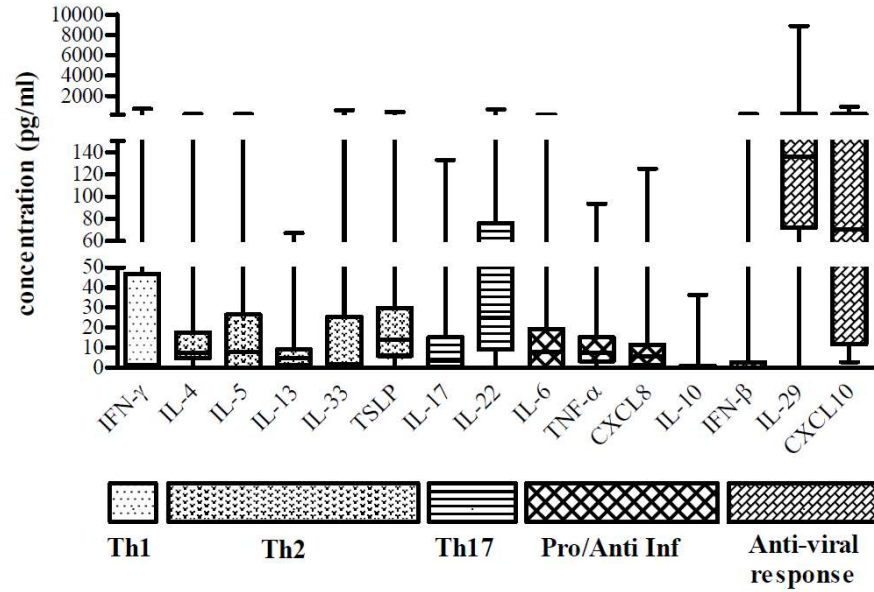
Supp TABLE II: Microbiological characteristics at exacerbation (T1) and at steady state (T2)

	Total population n=147	MTW† n=92	SIW‡ n=37	EVW§ n=18	Global p-value
Virus identification at T1 (PCR¶):	<i>n'</i> =146	<i>n'</i> =91	<i>n'</i> =37	<i>n'</i> =18	
At least one isolated virus	137 (93.8)	84 (91.3)	37 (100.0)	16 (94.1)	0.19
RV	106 (72.6)	64 (69.6)	30 (81.1)	12 (70.6)	0.41
RSV	11 (7.5)	7 (7.6)	2 (5.4)	2 (11.8)	0.72
Bocavirus	20 (13.7)	13 (14.1)	5 (13.5)	2 (11.8)	0.97
Enterovirus	11 (7.5)	7 (7.6)	3 (8.1)	1 (5.9)	1.00
Adenovirus	12 (8.2)	7 (7.6)	5 (13.5)	0 (0.0)	0.25
Influenza virus A or B	2 (1.4)	2 (2.2)	0 (0.0)	0 (0.0)	NA¶¶
Coronavirus	3 (2.1)	1 (1.1)	2 (5.4)	0 (0.0)	NA¶¶
Another virus	11 (7.5)	6 (6.5)	4 (10.8)	1 (5.9)	0.80
Virus identification at T2 (PCR¶):	<i>n'</i> =127	<i>n'</i> =80	<i>n'</i> =33	<i>n'</i> =14	
At least one isolated virus	85 (66.9)	52 (65.0)	23 (69.7)	10 (71.4)	0.83
RV††	63 (49.6)	37 (46.3)	18 (54.5)	8 (57.1)	0.61
RSV‡‡	6 (4.7)	3 (3.8)	1 (3.0)	2 (14.3)	NA
Bocavirus	15 (11.8)	9 (11.3)	3 (9.1)	3 (21.4)	0.54
Enterovirus	9 (7.1)	5 (6.3)	3 (9.1)	1 (7.1)	0.87
Adenovirus	14 (11.0)	9 (11.3)	2 (6.1)	3 (21.4)	0.27
Influenza virus A or B	2 (1.6)	2 (2.5)	0 (0.0)	0 (0.0)	NA¶¶
Coronavirus	12 (9.4)	7 (8.8)	3 (9.1)	2 (14.3)	0.73
Another virus	20 (15.8)	13 (16.3)	5 (15.2)	2 (14.3)	0.98

Values are reported as frequency (percentage). Comparisons between the three groups (MTW, SIW and EVW) were conducted using Chi-square test.

†MTW: Multiple-trigger wheeze; ‡SIW: Severe intermittent wheeze; §EVW: non severe intermittent wheeze; ¶PCR: Polymerase Chain Reaction; ††RV: Rhinovirus; ‡‡RSV: Respiratory Syncytial Virus; ¶¶NA: not available (n<8).

Blood



Induced sputum

