Neutrophilic Steroid-Refractory Recurrent Wheeze and Eosinophilic Steroid-Refractory Asthma in Children

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Abstract

Background: Little is known about inflammatory pathways of severe recurrent wheeze in preschool children and severe asthma in children.

Objectives: The aim of the "Severe Asthma Molecular Phenotype" (SAMP) cohort was to characterize phenotypes of severe recurrent wheeze and severe asthma during childhood in terms of triggers (allergic or not), involved cells (eosinophil or neutrophil) and corticoid responsiveness.

Methods: Children with moderate to severe asthma and preschool children with moderate to severe recurrent wheeze were enrolled prospectively. They underwent standardized clinical and blood work-up, and broncho-alveolar lavage (BAL) evaluation. Cluster analysis was applied to 350 children with 34 variables.

Results: Three clusters were identified; Cluster 1, *Neutrophilic steroid-refractory recurrent wheeze phenotype*, with 138 children uncontrolled despite high-dose inhaled corticosteroids (ICS) (92%, $p<0.001$), with more history of pneumonia (31%, $p<0.001$), more gastroesophageal reflux disease (37%, $p<0.001$) and the highest blood neutrophil count (mean 4.524cells/mm^3, $p=0.05$); Cluster 2*, Severe recurrent wheeze with sensitization to a single aeroallergen* (12%, $p=0.002$), with 104 children controlled with high-dose ICS (63%, $p<0.001$); Cluster 3, *Eosinophilic steroid-refractory asthma phenotype*, with 108 children uncontrolled despite high-dose ICS (76%, $p<0.001$) with more allergic rhinitis, atopic dermatitis and food allergies (82%, 40%, 31%, $p<0.001$, respectively). They also had a higher blood eosinophil count and a higher percentage of BAL eosinophil (506/mm^3, 2.6%, $p<0.001$ respectively).

Conclusion: Inflammation pathway of asthma and recurrent wheeze are related to eosinophil cells in older children and neutrophil cells in younger children. These results could improve personalized treatments.

What is already known about this topic?

In preschool children, recurrent wheezes have a good prognosis but severe phenotypes exist. At school age, severe asthma is often associated with multiple allergies. In these two cases, the physio-pathological pathways are not well known.

What does this article add to our knowledge?

Inflammatory cells and different triggers are associated with two phenotypes of severe obstructive diseases during childhood; neutrophils and bacterial infection in preschool children and eosinophils and multiple allergies at school age.

How does this study impact current management guidelines?

These two severe childhood obstructive diseases – neutrophilic steroid-refractory recurrent wheeze and eosinophilic steroid-refractory asthma – could be treated by targeted therapies such as antibiotic and Th2- biotherapy directed towards neutrophil and eosinophil inflammation respectively.

Key words:

Severe asthma, severe recurrent wheeze phenotypes, children, bacterial infection, gastro-esophageal reflux, multiple allergies

Abbreviations:

TAP: Trousseau Asthma Program, SAMP: Severe Asthma Molecular Phenotype, SARP: Severe Asthma Research Program, ISAAC: International Study of Asthma and Allergies in Childhood, ICS: inhaled corticosteroid, LTRA: leukotriene receptor antagonist, BMI: Body mass index, EVW: episodic viral wheeze, MTW: multiple triggers wheeze, GERD:

Gastroesophageal reflux, IgG: immunoglobulin G, IgA: immunoglobulin A, IgM: immunoglobulin M

Introduction

Asthma is a heterogeneous disease related to various phenotypes. Severe asthma is rare but represents a high burden of the disease. Two pathophysiological pathways of asthma can be described in terms of the principal triggers: allergic and non-allergic [1]. However, in 1999, Humbert *et al*. [2] found IgE in bronchial biopsies of patients testing negative for allergy. These results contest the dichotomous feature of *extrinsic* and *intrinsic* asthma. Moreover, it seems that in birth cohorts, allergic asthma conveys the highest risk of persistence during childhood [3]. Nevertheless, non-allergic asthma (with predominance in girls) seems to be more common and more severe than allergic asthma in cohorts of children suffering from asthma [4] than in adult patients [5, 6]. This difference could be due to the fact that the non-allergic asthma phenotype is not considered as a phenotype of asthma in the general population but related to viral-induced wheeze carrying with it the notion of a favourable disease course or remission during childhood.

The term “recurrent persistent wheeze” is applied to infants and preschool age children who present with recurrent episodes of coughing and/or wheezing. Although these symptoms are common in the preschool years, they are frequently transient [7]. Bacharier *et al.* [8] described a phenotype of severe intermittent wheezing in preschool children where children with oral corticosteroid used in the previous year were likely to have more severe disease, documented by a higher incidence of visits to the emergency room, hospitalizations and aeroallergen sensitization. Severe recurrent preschool wheeze is also defined by Fleming *et al.* [9] as a history of breathlessness and wheeze and persistent symptoms and/or frequent severe exacerbation despite a combination of high-dose ICS with a leukotriene receptor antagonist (LTRA). Although the underlying mechanisms are not yet known, some of these preschool children have evidence of airway remodelling and inflammation [10]. Some of them, especially those with severe recurrent wheeze, will also develop asthma during childhood

[11]. Indeed, a retrospective analysis of a cohort of severe asthma in children aged more than 6 years (Severe Asthma Research Program (SARP) cohort) suggests that many school-age children with severe asthma have symptoms that appeared within the first 24 months of life [12]. At the time of the development of various biotherapies directed against Th2 or Th1 pathways for the treatment of severe asthma [13], it could also be interesting to describe severe asthma in children and severe recurrent wheeze in preschool children (potentially at risk of persistence) as regards to the triggers and link to atopy, but also in terms of inflammatory features and corticoid responsiveness [11, 14, 15].

We therefore performed a study in a new population of the Trousseau Asthma Program (TAP) called the Severe Asthma Molecular Phenotype (SAMP) cohort, to characterize preschool recurrent wheeze phenotypes and severe asthma in children.

Methods

Design and setting

This was a prospective cross-sectional study performed from 2011 to 2015 from SAMP, a part of the TAP cohort, at Trousseau Hospital, Paris. All the children had been referred to the center by a primary care physician due to persistence of recurrent wheeze despite long-term treatment. Two-thirds of the children were from Paris and the surrounding area and the remaining third were from all regions of France. The Institutional Review Board of Saint Antoine Hospital, Paris, endorsed the protocol as an observational study. Written informed consent was obtained from the parents of the children included.

Inclusion criteria

The population included in the present study consisted of children meeting the following inclusion criteria: (1) children with severe asthma or severe recurrent wheeze (as defined below) aged from 1 to 15 years at the time of exploration; (2) who had undergone exploration at least 6 weeks after an episode of exacerbation, acute respiratory illness or treatment by antibiotics; (3) had a history of recurrent wheeze (more than three episodes of bronchodilator reversible bronchial obstruction documented within the previous 6 months); (4) had been explored by flexible bronchoscopy for severe asthma, severe recurrent wheeze, moderate recurrent wheeze or moderate asthma with unusual symptoms (i.e., cough with phlegm associated with wheezing, and/or persistent parenchymental shadowing); (5) for whom other diseases known to provoke wheezes had been ruled out by flexible bronchoscopy and broncho-alveolar lavage (BAL) with microbiological analysis consisting of bacterial cultures and viral analysis (PCR for influenza (A and B), parainfluenzae (1, 2, 3), metapneumovirus); available blood-cell count, IgG, IgM, IgA blood levels, post vaccine tetanus and diphtheria serologies; and a sweat test. In the rare cases of bronchiectasis and/or chronic severe, ear or sinus disorders, a bronchial biopsy/brushing was performed to rule out a primary ciliary

pathology. Definitions of asthma in children: (i) moderate asthma was defined as controlled asthma or partially controlled asthma with moderate dose ICS (≥200 and <500mcg/day fluticasone propionate (FP)) plus one other controller medication (leukotriene receptor antagonist (LRTA), long-acting beta agonist); (ii) severe asthma was defined as controlled asthma with high-dose ICS (≥500mcg/day FP) and two other controller medications and, (iii) steroid-refractory asthma was defined as uncontrolled asthma despite high-dose ICS (≥500mcg/day FP) and two other controller medications according to GINA guidelines [16] and dosage for fluticasone available in France.

Definition of asthma in preschool children: (i) moderate recurrent wheeze was defined as controlled or partially controlled symptoms with moderate-dose ICS (≤200mcg/day FP) and LRTA (10); (ii) severe recurrent wheeze was defined as controlled symptoms with high-dose ICS (>200 mcg/day) and LRTA and, (iii) steroid-refractory recurrent wheeze was defined as uncontrolled symptoms (persistent and frequent exacerbations) despite high-dose ICS (>200 mcg/day) and LRTA [9]. The severity of asthma or recurrent wheeze of the entire population was assessed after at least 6 months of follow up prior to inclusion in the study, by an experienced pulmonologist paediatrician after repeated individual or group health education measures to improve adherence to anti-asthmatic treatment and after advice by an environmental specialist to reduce exposure to indoor biological pollutants.

Totally controlled asthma was defined as any nocturnal or daily symptoms, any exacerbation, any use of short acting B2 agonist, any activity limitation due to asthma and partially or uncontrolled asthma as presence of 1-2 or 3-4 presence of previous parameters respectively [16].

Health outcomes were collected in a computerized database using questionnaires and clinical exams following a standardized protocol. Gender, body mass index (BMI) (weight/height^2) and ethnic group and parents' socio-professional categories were recorded.

175 The age of wheeze onset was defined as the time of the first episode of obstructive symptoms.
176 Children were classified as having either episodic viral wheeze (EVW) (wheezing only during
177 colds and remaining asymptomatic between episodes) or multiple trigger wheeze (MTW)
178 (wheezing during colds but symptomatic between episodes with wheezing activated by dust,
179 grass, pets, tobacco smoke, exercise or cold air).

180 ***Atopic status***

181 The maternal and paternal history of asthma was collected. Allergic rhinitis and active atopic
182 dermatitis were assessed by questions from the International Study of Asthma and Allergies
183 in Childhood (ISAAC) [17] and IgE-mediated food allergy was defined by relevant symptoms
184 of food allergy within the 6 hours following consumption of the food allergen associated with
185 an allergic sensitization to the same allergen [18].

186 Standard skin prick test (SPT), Phadiatop Infant test® and serum specific IgE levels for
187 current allergens and staphylococcal toxins were assessed (*see* online supplement). We
188 defined sensitization as positive SPT as well as positive specific IgE to ensure an accurate
189 diagnosis. Perennial sensitization only was defined as sensitization to house dust mite and/or
190 cat or dog dander without seasonal sensitization. Sensitization to grass and/or birch pollens
191 only, with no perennial sensitization, defined seasonal sensitization.

192 ***Comorbidities***

193 Gastroesophageal reflux disease (GERD) was defined as the presence of suggestive
194 symptoms or pathological 24-hour oesophageal pH-metry or good response to therapy [19].

195 A history of pneumonia was defined as a medical diagnosis of a history of fever and cough or
196 breathing distress associated with alveolar syndrome on chest X-ray [20]. The number of
197 antibiotic treatments for pneumonia history in the previous year was noted and classified into
198 three categories: children having (1) 0 to 3 treatments, (2) 3 to 12 treatments and (3) more
199 than 12 treatments.

200 ***Flexible bronchoscopy***

201 Flexible bronchoscopy was performed under local anaesthesia as previously described in our
202 center in which more than 300 flexible bronchoscopies are routinely performed a year [21].
203 Children received intra-rectal midazolam and atropine with nitrous oxyde (Entonox) during
204 the bronchoscopy. Broncho-alveolar lavage (BAL) was collected [22] and processed for
205 cytology by the hospital cytopathology department.

206 ***Microbiological cultures of BAL***

207 Aliquots of BAL were systematically used for microbiological assessment with bacterial
208 cultures and viral analysis (PCR for influenza (A and B), parainfluenza (1,2,3),
209 metapneumovirus). Positive bacterial culture was defined as positive if growth was $\geq 10^4$
210 colony-forming unit (CFU)/ml [23],

211 ***Inflammatory markers***

212 Blood eosinophilia and serum total IgE were collected. Non-specific blood inflammatory
213 markers included neutrophil cell count and serum IgG, IgA and IgM levels (*see* online
214 supplement). Eosinophil and neutrophil cells in BAL were expressed as a percentage of the
215 total cell count.

216 ***Statistical analysis***

217 Statistical analysis was performed with R version 2.12.0 (https://www.r-project.org) (see
218 online supplement). The 34 selected variables were analyzed using an unsupervised
219 classification approach to find groups of patients in the study population. The variables were
220 (1) age at the time of exploration; (2) age of asthma onset; (3) $>75^{th}$ percentile BMI; (4)
221 gender; (5) parents' socio-professional categories; (6) ethnic group; (7) maternal history of
222 asthma; (8) paternal history of asthma; (9) history of pneumonia; (10) history of GERD; (11)
223 active allergic rhinitis; (12) active atopic dermatitis; (13) IgE-mediated food allergy; (14)
224 more than two hospitalizations for severe exacerbation during the previous year; (15)

wheezing triggers; (16) annual number of antibiotics for pneumonia; (17) asthma severity; (18) perennial sensitization only; (19) seasonal sensitization only; (20) both sensitization to perennial and seasonal allergens; (21) sensitization to egg and/or cow's milk proteins; (22) peanut sensitization; (23) *Alternaria alternata* sensitization; (24) staphylococcal toxin sensitization; (25) Phadiatop Infant test® $\geq$0,35KU/l; (26) absolute value of blood neutrophil; (27) absolute value of blood eosinophil; (28) serum total IgE (kU/L); (29) IgG level (g/L); (30) IgA level (g/L); (31) IgM level (g/L); (32) BAL neutrophil percentage; (33) BAL eosinophil percentage; (34) BAL microbiological cultures.

A hierarchical bottom-up clustering was applied using the R package 'cluster'[24]. Gower distance was considered since quantitative and categorical variables are involved in the cluster analysis. By definition, this distance implies that standardization is applied to each variable, avoiding scaling bias. The Ward's linkage which minimize the within cluster sum of squares was considered [25]. The number of clusters is chosen at the end of the procedure using the scree plot of the distance of merging clusters. In our case, three clusters clearly emerged. The dissimilarity matrix is then visualized in a low-dimensional space (Figure 1) using a Classical multidimensional scaling [25]. Besides, a factor analysis for mixed data [26] was applied to identify the variables involved in the first two principal components. For each variable, the difference between clusters was evaluated using ANOVA and Kruskal–Wallis test for continuous variables and $\chi 2$ test and Fisher exact test for categorical variables.

Results

Patients

The study initially considered 355 children for inclusion. Of these, five were not retained for the following reasons: (1) two had a lung malformation, (2) two had positive viral analysis in BAL and, (3) more than three parameters were missing for the cluster analysis for one. Thus, the final study population consisted of 350 children, 237 (64.4%) of whom were boys. Twenty-eight children have missing BAL cells data (Table 1). No severe complication was detected after the bronchoscopy. Transient desaturation was observed in 10 children. Detailed characteristics of the entire population (preschool children aged less than 72 months and children aged more than 72 months) are shown in Table 1.

Asthma phenotypes according to cluster analysis

Cluster analysis produced a dendogram (Figure 2) and a scatter plot (Figure 1) revealing three clusters of children with shared phenotypic characteristics (Tables 2 to 4). Factor analysis indicates that Age, Eosinophils and Phadiatop Infant Test play an important role for principal component 1 whereas asthma control and asthma severity are main factors involved in principal component 2. Figure 3 represents blood cell inflammation and severity of asthma according to the three clusters.

Cluster 1

This cluster comprised 138 children (Table 2) with early onset of wheeze at an average age of 6.8 months ($p<0.001$) (Table 2). Severe recurrent wheeze represented 94% of the cluster ($p<0.001$) and was uncontrolled despite high-dose ICS (92%, $p<0.001$) (Table 2). Wheezing triggers were principally viral induced (75%, $p<0.001$) (Table 2). These children had more non-allergic comorbidities such as history of pneumonia (31%, $p<0.001$) and were more likely to have a history of GERD (37%, $p<0.001$) than children of the other clusters (Table 3). The BAL bacterial cultures were significantly more positive (26%, $p<0.001$) in this cluster, with a predominance of *Haemophilus influenzae* and *Branhamella catarrhalis* (Table 3). Thirty percent of the children in this cluster had more than four antibiotic treatments annually ($p<0.001$) (Table 3). This phenotype was mostly non-allergic with a negative Phadiatop Infant Test® in 85% of the cases ($p<0.001$) (Table 3). It was also associated with the highest blood neutrophil count (mean 4.524 cells/mm^3, $p=0.05$) (Table 4) and can be summarized as "*Neutrophilic steroid-refractory recurrent wheeze phenotype"*

Cluster 2

This cluster comprised 104 children with an average age of 4.7 years (Table 2). This phenotype was severe and controlled with high-dose ICS in the majority of cases (63%, $p<0.001$) (Table 2). Phadiatop Infant Test® was positive in 34% of the cases with sensitization to aeroallergens only (12%, $p=0.002$) (Table 3). These children, as in cluster 1, were more likely to have a history of pneumonia (31%, $p<0.001$) compared to cluster 3 (Table 3). The children in this cluster were also more likely to have attended day-care facilities before 3 years (42%, $p<0.001$) compared to the other clusters (*see e-table 1 in online supplement*). This cluster can be summarized as "*Severe recurrent wheeze with sensitization to a single aeroallergen"*

Cluster 3

This cluster comprised 108 children who were significantly older (with a mean age of 10.9 years) and had a higher BMI (above the 75th percentile) compared to the other clusters (35%, $p<0.001$) (Table 2). Asthma was uncontrolled despite high-dose ICS in the majority of cases (76%, $p<0.001$) compared to cluster 2 (Table 2). Paternal asthma was more frequently encountered in this cluster (Table 2). Wheezing triggers were multiple (66%, $p<0.001$) (Table 2). This cluster was characterized by more allergic rhinitis (82%, $p<0.001$) that was severe in 32% of cases ($p<0.001$), more active atopic dermatitis (40%, $p<0.001$) and more IgE-mediated food allergies (31%, $p<0.001$) (Table 3). Phadiatop Infant Test® was positive in 98% of the cases ($p<0.001$) with both perennial and seasonal sensitization in 52% ($p<0.001$) (Table 3). These children had more sensitization to *Alternaria alternata* (29%, $p<0.001$) (Table 3) and staphylococcal toxins (53%, $p<0.001$) (Table 3 and *see* e-table 2 in online supplement). Blood atopy markers were higher in this group with a higher total-IgE (mean 1086 kU/L, $p<0.001$), higher blood eosinophil count (mean 506/mm^3, $p<0.001$) and higher percentage of eosinophils in BAL (mean 2.6%, $p<0.001$) (Table 4) but also higher IgG and IgA levels (values equal to or above the 75th percentile in 52 and 54% of cases respectively, $p<0.001$). This phenotype can be summarized as *"Eosinophilic steroid-refractory asthma phenotype"*.

Discussion

Interpretation of findings

This article presents the detailed clinical and biological characteristics of a cohort of 350 preschool and children with both severe and moderate asthma or recurrent wheeze with BAL evaluation. To date, in the literature, only one European cohort of 298 children with severe or mild/moderate asthma and recurrent preschool wheeze has been described [9] in which the main result was impaired quality of life related to the severity of the disease. The main result of our study is that two phenotypes of severe asthma and severe recurrent wheeze are related to different inflammatory cells according to the allergic or non-allergic nature of the comorbidities. More specifically, comorbidities are related to: (1) a history of pneumonia or a history of GERD in younger children with steroid-refractory recurrent wheeze and, (2) multiple allergic comorbidities (especially atopic dermatitis) in steroid-refractory asthma in children. Dual cellular inflammation in the blood and BAL is related to the principal trigger, i.e., neutrophilic in non-allergic steroid-refractory recurrent wheeze associated with a history of pneumonia in preschool children and eosinophilic in allergic steroid-refractory asthma with allergic triggers in children, as previously described [27].

"*Neutrophilic steroid-refractory recurrent wheeze phenotype"*

This phenotype is in accordance with other studies that found neutrophilic cells in BAL in infants with recurrent wheeze [28, 29]. Several studies are also in accordance with the association of neutrophilic inflammation, bacterial infections and wheeze, as seen in the children of our cluster 1. Neutrophilic asthma represents up to 25% of symptomatic asthma patients and 59% of patients on high-dose ICS [30]. However, to date, this phenotype has only been described in adult populations, mainly with late onset asthma, rather than in children. Rackemann [6] first recognized the possible importance of respiratory infections on the aetiology of *intrinsic* (non-allergic) asthma over 60 years ago. Pathogen-host interactions

have been demonstrated to lead to bronchial inflammation in asthma [31]. Therefore, the term *intrinsic* asthma can be of clinical relevance and raises the possibility that these respiratory infections may play an important role in this asthma phenotype. Viral respiratory tract infections weaken local defences against opportunistic bacterial pathogens, enhancing the risk of secondary pathogens broaching mucosal barriers. The result is an amplification of immune-inflammatory responses associated with tissue damage [32]. Thus, in an Australian cohort, both the respiratory syncytial virus and rhinovirus were independently associated with acute respiratory illness symptom expression, and after controlling for virus, *Haemophilus, Streptococcus*, and/or *Moraxella* microbial profile groups remained highly significantly associated with acute respiratory illness [33]. More specifically, a predominance of *M. catarrhalis* or members of the *Haemophilus* or *Streptococcus* genera was found to be associated with a neutrophilic airway phenotype in treatment-resistant persistent asthma. In this particularly severe asthma phenotype, innate immune mechanisms may lead to a shift towards Th_1 or Th_{17} mediated neutrophilic inflammation [34]. This pulmonary neutrophilic inflammation is corticosteroid-refractory, as in patients with severe asthma. More recent studies [35, 36] provide clinical support to our data, suggesting that the airway microbiota in non-eosinophilic (neutrophilic) asthma is different from eosinophilic phenotypes, and may be manipulated to improve clinical outcomes.

In our cluster 1, GERD was a significant non-allergic comorbidity associated with steroid-refractory recurrent wheeze that has been well described in literature. The prevalence of GERD symptoms in patients with asthma is around 60% [37]. In adult populations, a higher incidence of GERD [38] is associated with frequent hospitalizations, poor respiratory function and late onset disease in non-allergic asthmatic patients. In the SARP cohort [39], GERD with low pH was also independently associated with high BAL neutrophil counts. A chronic, undetected infection could also play a role in the neutrophilic inflammation and low pH [40].

These subpopulations may be responsive to targeted, steroid-sparing therapy, e.g., anti-acid therapy and/or exercise and weight loss. In our study, GERD was encountered in the cluster with younger children with frequent hospitalizations and neutrophil inflammation in the blood and BAL. This is the first time this phenotype, very similar to an adult phenotype, has been described in children. In a previous article [10], we describe a severe non-atopic phenotype, with a special trajectory during the preschool period: half of the children in this phenotype progress to moderate to severe allergic asthma and half to mild or episodic asthma. We thus believe that this recurrent severe wheeze phenotype is a particular phenotype that does not necessarily have a good prognosis. Nevertheless, children of cluster 2 will probably be at risk of persistent asthma during childhood due to the link of this cluster to atopy defined by the association to allergic sensitization [10].

"Eosinophilic steroid-refractory asthma phenotype"

An earlier study by our group in asthmatic children showed a link between intra-alveolar eosinophilia and atopy [27]. Moreover, it is known that blood eosinophilia is closely correlated to eosinophil inflammation in the deep lung tissues [41]. We also found this relationship between broncho-alveolar and blood eosinophilia in the present study. Hargreaves [42] showed that measurements of sputum eosinophils can be used to guide effective corticosteroid treatment in asthma. Nevertheless, in the case of multiple allergic sensitization and/or multiple allergic comorbidities (such as atopic dermatitis or food allergy) or particular sensitization (such as staphylococcal enterotoxin), high levels of a marker of eosinophilic inflammation potentially associated with the steroid-refractory asthma phenotype have been found [43]. Therefore, serum staphylococcal enterotoxin-specific IgE levels have been positively associated with the severity of asthma [44, 45] particularly in late onset asthma [46], linked to staphylococcal serine protease-like proteins [47] as "pacemakers" of

allergic airway reactions to staphylococcus aureus. It indirectly suggests a role of this pathogen in the aetiology of airway disease and implies that pathogen-specific IgE production and allergic-type inflammation may occur with a chronic inflammatory process.

Severe acute asthma requiring intensive care is more frequently associated with food allergy [48]. Mould sensitization in allergic asthma is associated with severe exacerbations requiring hospitalization and uncontrolled asthma despite high doses of ICS [49]. Recently, a cluster analysis applied to patients with near fatal asthma [50], described three clusters including one phenotype related to sensitization to soybean or *Alternaria alternata,* thereby suggesting the existence of a specific pathogenic mechanism of these particular sensitizations. This cluster is very close to our cluster 3 called *"Eosinophilic steroid-refractory asthma phenotype"* in which sensitization to *Alternaria alternata* was encountered in 29% ($p<0.001$) of the children. In the TAP cohort, we have already described [51] that multiple allergies, defined as sensitization to both food and inhaled allergens, define a severe phenotype of asthma in terms of severity of exacerbations. Moreover, the severe allergic phenotype with atopic dermatitis is strongly associated with eosinophil-driven inflammatory markers. Recently, in a cluster analysis of 125 children, our team described four clusters [51]. Among them, one cluster called *"Multiple allergies and severe asthma"* was characterized by atopic dermatitis in 100% of the cases, higher values of total-IgE (1123 kU/L) and high fractional exhaled nitric oxide (median value 67 ppb). This phenotype is also very close to the present cluster 3 "*Eosinophilic steroid-refractory asthma phenotype"* in which atopic dermatitis is present in 43% of the cases ($p<0.001$). In the two allergic clusters of the present study, multiple trigger wheeze is highly prevalent. This relation between allergy and multiple triggers has been described previously [52]. Furthermore, this association has been highlighted in a study of 551 much younger children enrolled in the TAP [4]. We described an atopic multiple trigger

wheeze phenotype including more children with multiple trigger wheeze (68%) than the other two clusters with atopic dermatitis in 75% of cases and positive Phadiatop Infant test® results in 90% of the cases. A history of paternal asthma was more frequently encountered in this cluster as a genetic component of allergic asthma [53].

Strengths and limits of the study

The strength of the present study is that it was performed in a large and well-defined population of children with severe asthma or severe recurrent wheeze. To our knowledge, it is the first study of a cohort of children with severe recurrent preschool wheeze and severe school-age asthma, analysed in terms of clinical, biological and bronchial examinations.

The main limitation of our study lies in the fact that all the patients were recruited from one center. Nevertheless, while two-thirds of the asthmatic children were from Paris and the surrounding area (>10 million inhabitants), the remaining one-third live in regions throughout France, which limits this potential bias. A second limitation is that cluster analysis is an unsupervised analysis where the "statistical machine" clustered the data by age. Nevertheless, our results are in accordance with our previous phenotypes in preschool children [4] and in children [54]. Thirdly, the two-dimensional representation should be carefully interpreted since a relatively small part of the total variance is explained by the first two components. Finally, another limitation is that the study was cross-sectional. However, the longitudinal assessment of this SAMP cohort is ongoing, and the stability of the phenotypes we describe here will be analysed with a longer follow up in a future publication.

Conclusion

From this large study of children suffering from severe asthma or severe recurrent wheeze, we describe two steroid-refractory phenotypes. We suggest that these phenotypes could be included in recommendations for managing severe asthma and severe recurrent wheeze in children. To optimize treatment strategies, anti-allergic drugs such as anti-IgE (omalizumab) or anti-interleukin-5 could be administered to children with the phenotype *"Eosinophilic steroid-refractory asthma phenotype* whereas anti-neutrophil drugs, such as macrolides [55] and anti-acid therapy could be an appropriate treatment choice for children with the phenotype "*Neutrophilic steroid-refractory recurrent wheeze phenotype"*.

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Figure 1: Scatter plot of the first 2 principal coordinates, which display the 3 clusters identified with Ward's method (n=350). The 2 principal coordinates obtained captured 12% and 7% of the total variance. Each point represents a single subject. The plot depicts clustering and clear separation of children with neutrophilic steroid-refractory recurrent wheeze in young children (n=138) (blue circles), severe recurrent wheeze with sensitization to aeroallergen only in children (n=104) (red circles), and eosinophilic steroid-refractory asthma with multiple allergies in children (n=108) (green circles).

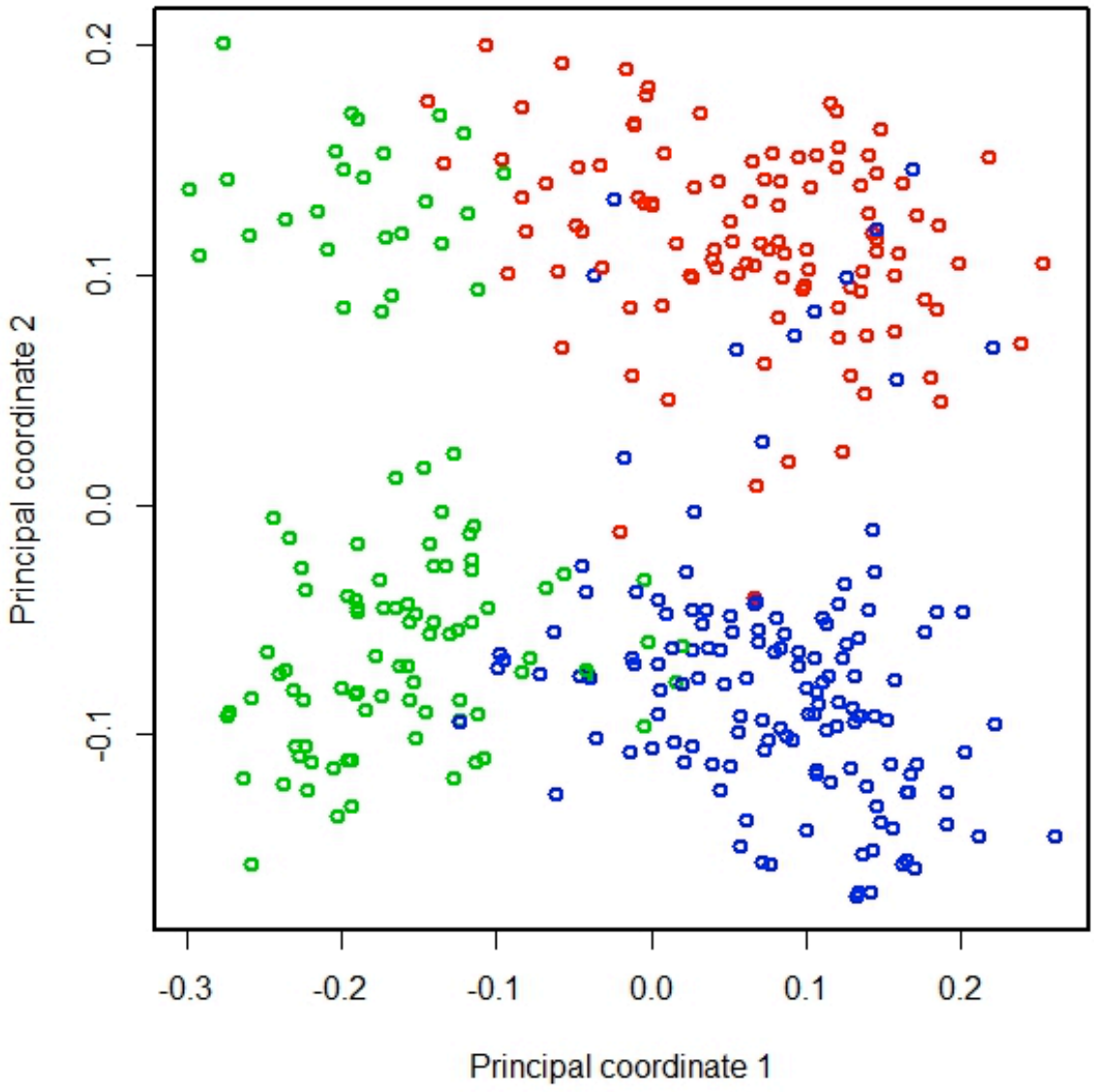

Figure 2: Dendrogram for the entire population (n=350) obtained by using a hierarchic bottom-up clustering method. Three clusters emerged.

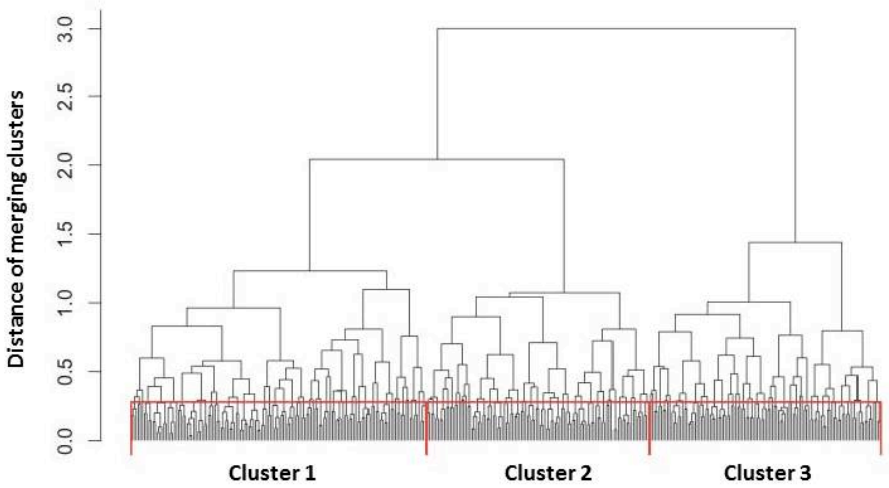

Figure 3: Representation of the three clusters according to blood neutrophils, blood eosinophils and percentage of refractory asthma for the entire population (n=350).

Blood neutrophils and eosinophils are expressed in cells/mm^3.

Circled areas represent the percentage of steroid-refractory asthma or steroid-refractory recurrent wheeze defined as the percentage of severe asthma or severe recurrent wheeze, uncontrolled with high doses of inhaled corticosteroids.

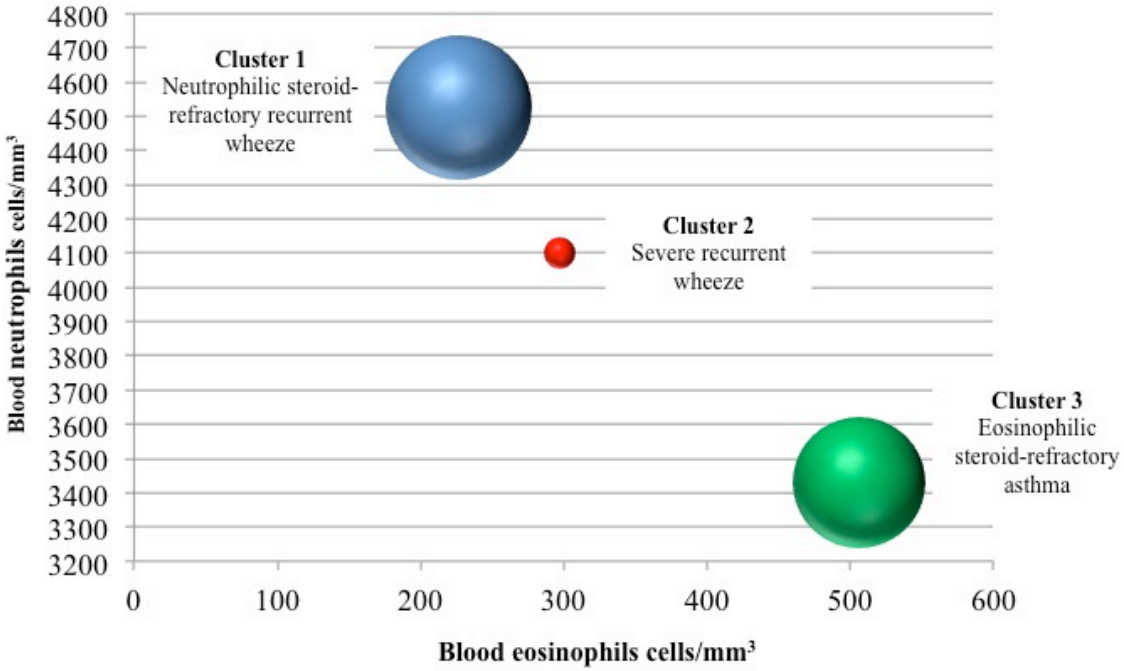

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Table 1: Patients' characteristics according to the entire population

	Preschool recurrent wheeze n= 217	Asthma in children n=133	p-value
Demographic details			
Gender, male n (%)	148 (68)	89 (66)	0.89
Age, months mean (SD)	33.9 (21.9)	138 (22.5)	p<0.01
Exposure to tobacco n (%)	80 (36)	38 (29)	0.14
Low socio-economic categories of parents n (%)	96 (44)	60 (45)	0.62
Ethnic origin n (%)			0.01
Caucasian	172 (79)	92 (69)	
African	43 (20)	32 (24)	
Indian	2(1)	8(6)	
Asian	0 (0)	1 (1)	
Anthropometry			
BMI n, % (SD)	16.4 (2.0)	19 (4)	p<0.01
Personal atopy			
Atopic Dermatitis n (%)	68 (31)	64 (48)	0.002
Allergic Rhinitis n (%)	71 (33)	92 (69)	p<0.01
IgE-mediated food allergy n (%)	26 (12)	27 (20)	0.08
Familial atopy			
Maternal asthma n (%)	53 (24)	30 (22)	0.75
Paternal asthma n (%)	40 (18)	30 (22)	0.45
Asthma history			
Age at wheeze onset months mean (SD)	7.7 (12.5)	19.9 (25.0)	p<0.01
Single trigger wheeze n (%)	156 (72)	59 (44)	p<0.01
Multiple trigger wheeze n (%)	61 (28)	74 (55)	p<0.01
>2 hospitalizations for severe exacerbation n (%)	37 (17)	10 (7)	0.01
Asthma severity			0.37
Moderate n (%)	34 (16)	26 (20)	
Severe n (%)	183 (84)	107 (80)	
Severe asthma control			0.16
Controlled with high-dose ICS n (%)	56 (26)	47 (35)	
Uncontrolled with high-dose ICS n (%)	127 (59)	86 (65)	
Blood inflammation			
Blood PN mean (SD)	4429 (2912) *(n=213)*	3460 (2098) *(n=122)*	p<0.01
Blood PE mean (SD)	273 (325) *(n=213)*	432 (378) *(n=122)*	p<0.01
IgG level mean (SD)	6.9 (2.2) *(n=188)*	9.9 (2.2) *(n=122)*	p<0.01
IgA level mean (SD)	0.6 (0.4) *(n=188)*	1.5 (0.7) *(n=122)*	p<0.01
IgM level mean (SD)	1.0 (0.8) *(n=188)*	1.0 (0.5) *(n=122)*	p<0.01
BAL cells			
BAL PN mean (SD)	7.0 (10.6) *(n=209)*	4.9 (7.5) *(n=113)*	0.01
BAL PE mean (SD)	0.7 (2.2) *(n=209)*	2 (5.5) *(n=113)*	p<0.01
Allergic sensitization			
Positive Phadiatop Infant Test® n (%)	59 (27) *(n=214)*	102 (77) *(n=132)*	p<0.01
Perennial sensitization only n (%)	30 (14) *(n=214)*	1 (1) *(n=132)*	p<0.01
Seasonal sensitization only n (%)	2 (1) *(n=214)*	3 (2) *(n=132)*	1
Perennial and seasonal sensitization n (%)	3 (1) *(n=214)*	52 (39) *(n=132)*	p<0.01
Alternaria alternata sensitization n (%)	1 (0.4) *(n=214)*	30 (22) *(n=132)*	p<0.01
Peanut sensitization n (%)	6 (3) *(n=214)*	20 (15) *(n=132)*	p<0.01
Cow's milk and/or egg sensitization n (%)	28 (13) *(n=214)*	25 (21) *(n=132)*	0.19
Staphylococcal toxins sensitization n (%)	17 (9) *(n=191)*	46 (35) *(n=121)*	p<0.01
Non-allergic comorbidities			
History of pneumonia n (%)	68 (31)	20 (15)	p<0.01
GERD n (%)	85 (39) *(n=214)*	29 (22) *(n=133)*	p<0.01
BAL microbiology	*(n=215)*	*(n=131)*	
Negative n (%)	158 (73)	112 (84)	p<0.01
Positive n (%)	57 (26)	19 (14)	

Definition of abbreviation: BMI, body mass index; ICS, inhaled corticosteroid, IgG, immunoglobulin G; IgA, immunoglobulin A; Ig M, immunoglobulin M; blood PN, blood neutrophil cells; blood PE, blood eosinophil cells; BAL: broncho-alveolar lavage; GERD, gastro-esophageal reflux disease

Table 2: **Patients' and asthma characteristics according to cluster analysis**

	Entire population n=350	**Cluster 1** Neutrophilic steroid-refractory recurrent wheeze phenotype **n= 138**	**Cluster 2** Severe recurrent wheeze with sensitization to a single aeroallergen **n= 104**	**Cluster 3** Eosinophilic steroid-refractory asthma phenotype **n= 108**	**p value***
Patient's characteristics					
Gender, male n (%)	237 (68)	98 (71)	72 (69)	67 (62)	0.30
Age, months mean (SD)	73.6 (57.8)	40.6 (39.2)	57.3 (39.8)	**131.3 (49.0)**	<0.001
BMI† n (%)	88 (25)	22 (16)	14 (13)	**52 (35)**	<0.001
Low socio-economic categories of parents n (%)	156 (46)	59 (44)	53 (53)	44 (41)	0.51
Ethnic origin (n, %)					0.07
Caucasian	264 (75)	113 (82)	74 (71)	77 (71)	
African	75 (21)	22 (16)	28 (27)	25 (23)	
Indian	10 (3)	3 (2)	1 (1)	6 (6)	
Asiatic	1 (0.3)	0 (0)	1 (1)	0 (0)	
Maternal asthma n (%)	83 (24)	33 (24)	20 (19)	30 (28)	0.36
Paternal asthma n (%)	70 (20)	28 (20)	13 (13)	**29 (27)**	0.03
Asthma characteristics					
Age at wheeze onset, months mean (SD)	12.3 (19.3)	**6.8 (6.9)**	11.6 (19.7)	19.9 (26.0)	<0.001
Moderate asthma or moderate recurrent wheeze n (%)	60 (17)	8 (6)	**37 (36)**	15 (14)	<0.001
Severe asthma or severe recurrent wheeze n (%)	290 (83)	**130 (94)**	67 (64)	93 (86)	
Controlled asthma or controlled recurrent wheeze with high-dose ICS n (%)	77 (22)	3 (2)	**63 (61)**	11 (10)	<0.001
Uncontrolled asthma or uncontrolled recurrent wheeze with high-dose ICS n (%)	213 (61)	**127 (92)**	4 (4)	82 (76)	
> 2 hospitalizations for severe exacerbation n (%)	47 (13)	24 (17)	11 (11)	12 (11)	0.21
Triggers n (%)					<0.001
EVW	215 (61)	103 (75)	75 (72)	37 (34)	
MTW	135 (39)	35 (25)	29 (28)	71 (66)	

Definition of abbreviation: BMI, body mass index, ICS, inhaled corticosteroid; EVW, episodic viral wheeze; MTW, multiple trigger wheezes

Boldface values indicate statistical significance

*ANOVA or χ^2 test when conditions were respected, Kruskal-Wallis or Fisher exact test otherwise

†BMI is expressed as number of children with values equal to or above the 75th percentile

Table 3: Allergic and non-allergic comorbidities according to cluster analysis

	Entire population n=350	**Cluster 1** Neutrophilic steroid-refractory recurrent wheeze phenotype **n= 138**	**Cluster 2** Severe recurrent wheeze with sensitization to a single aeroallergen **n= 104**	**Cluster 3** Eosinophilic steroid-refractory asthma phenotype **n= 108**	p value *
Allergic comorbidities					
Active atopic dermatitis n (%)	79 (23)	25 (18)	11 (11)	**43 (40)**	<0.001
IgE-mediated food allergy n (%)	53 (15)	15 (11)	5 (5)	**33 (31)**	<0.001
Allergic rhinitis n (%)					
Presence	161 (46)	37 (27)	35 (34)	**89 (82)**	<0.001
Severe	59 (17)	19 (4)	6 (6)	**34 (32)**	
Total-IgE mean (SD)	405 (930)	64 (4)	129 (29)	**1086 (869)**	<0.001
Phadiatop Infant Test® n (%)	161 (46)	20 (15)	35 (34)	**106 (98)**	<0.001
Perennial sensitization only§ n (%)	69 (20)	2 (6)	16 (39)	**51 (48)**	<0.001
Seasonal sensitization only‖ n (%)	5 (1)	1 (3)	**4 (12)**	0 (0)	0.002
Perennial and seasonal sensitization** n (%)	55 (3)	3 (11)	2 (6)	**50 (52)**	<0.001
Alternaria alternata sensitization n (%)	31 (9)	2 (6)	3 (9)	**26 (29)**	<0.001
Peanut sensitization n (%)	26 (7)	4 (12)	3 (10)	19 (22)	0.25
Cow's milk and/or egg sensitization n (%)	53 (15)	11 (32)	15 (44)	27 (30)	0.31
Staphylococcal toxins sensitization n (%)	63 (18)	8 (6)	4 (4)	**50 (53)**	<0.001
Non allergic comorbidities					
GERD n (%)	**112 (32)**	**64 (37)**	27 (26)	21 (20)	<0.001
History of pneumonia n (%)	88 (25)	**43 (31)**	33 (31)	12 (11)	<0.001
Annual antibiotic treatments n (%)					<0.001
0-3	274 (78)	96 (70)	79 (76)	99 (92)	
4-12	56 (16)	**28 (20)**	23 (22)	5 (5)	
>12	20 (6)	**14 (10)**	2 (2)	4 (4)	
BAL microbial cultures† n (%)					<0.001
Positive bacterial culture	76 (22)	**36 (26)**	22 (21)	18 (17)	
Haemophilus influenzae‡	18 (5)	8 (6)	9 (9)	1 (1)	
Staphylococcus aureus‡	13 (4)	3 (2)	1 (1)	9 (8)	
Streptococcus pneumoniae‡	11 (3)	4 (3)	4 (4)	3 (3)	
Branhamella catarrhalis‡	18 (5)	12 (9)	4 (4)	2 (2)	
Other bacteria	16 (5)	9 (6)	4 (4)	3 (3)	

Definition of abbreviations: IgE, immunoglobulin E; Total-IgE, total immunoglobulin E; GERD, gastro-esophageal reflux disease

Boldface values indicate statistical significance

* χ^2 test when conditions were respected or Fisher exact test otherwise

§ Perennial sensitization was defined as IgE sensitization to house dust mite and/or cat or dog dander without seasonal sensitization

‖ Seasonal sensitization was defined by unique sensitization to grass pollens and/or birch pollens without perennial sensitization

**Perennial and seasonal sensitization was defined as IgE sensitization to house dust mite and/or cat or dog dander with seasonal sensitization

Specific IgE values were positive if ≥0.35 kU/L

† Total immunoglobulin E is expressed in kU/L

† Positive bacterial culture was defined as growth ≥10^4 colony-forming unit (CFU)/ml

Table 4: Blood and broncho-alveolar lavage inflammatory markers according to cluster analysis

	Entire population n=350	**Cluster 1** Neutrophilic steroid-refractory recurrent wheeze phenotype **n= 138**	**Cluster 2** Severe recurrent wheeze with sensitization to a single aeroallergen **n= 104**	**Cluster 3** Eosinophilic steroid-refractory asthma phenotype **n= 108**	**p value** *
Blood PN† mean (SD)	4058 (2669)	**4524 (3152)**	4100 (2640)	3430 (1784)	0.05
Blood PE† mean (SD)	334 (355)	227 (221)	297 (422)	**506 (359)**	<0.001
BAL PN§ mean (SD)	6.2 (9.6)	6.9 (10.1)	6.5 (9.5)	5.3 (9.0)	0,10
BAL PE§ mean (SD)	1.2 (3.9)	0.5 (2.1)	0.6 (1.7)	**2.6 (6.1)**	<0.001
IgG‡ n (%)	79 (25)	10 (8)	17 (19)	**52 (52)**	<0.001
IgA‡ n (%)	79 (25)	16 (13)	9 (10)	**54 (54)**	<0.001
IgM‡ n (%)	78 (25)	25 (21)	22 (25)	**31 (31)**	0.21

Definition of abbreviations: blood PN, blood neutrophil cells; blood PE, blood eosinophil cells; BAL, broncho-alveolar lavage; IgG, immunoglobulin G; IgA, immunoglobulin A; Ig M, immunoglobulin M;
Boldface values indicate statistical significance

* ANOVA or χ^2 test when conditions were respected, Kruskal-Wallis or Fisher exact test otherwise

†Blood neutrophil absolute count and blood eosinophil absolute count are expressed as number of cells /mm^3

§Broncho-alveolar neutrophil and eosinophil cells are expressed as percentage of total broncho-alveolar cell count

‡ IgG, IgA, IgM are expressed as number of children (percentage) with values equal to or above the 75th percentile

ONLINE REPOSITORY

MATERIALS AND METHODS

Study design

Exclusion criteria were (i) chronic obstructive pulmonary disease (congenital or acquired origin) other than asthma (ii) prematurity and (iii) an exacerbation or acute respiratory illness at the time of exploration.

Atopic status

The maternal and paternal history of asthma was collected. Allergic rhinitis and active atopic dermatitis were assessed by questions from the International Study of Asthma and Allergies in Childhood (ISAAC) [E1] and IgE-mediated food allergy was defined by relevant symptoms of food allergy within the six hours following food allergen's consumption associated with an allergic sensitization to the same allergen [E2].

Biological markers of atopy were measured in peripheral blood. These included (1) less-specific markers such as blood eosinophilia (cell counting by automated Sysmex; France): (2) total-IgE (measured by ImmunoCAP®; Phadia, Uppsala, Sweden); (3) allergenic sensitization to aeroallergens and food allergens defined as a positive skin-prick test ((SPT) (wheal allergen ≥3 mm in the absence of a positive reaction to the negative control), and confirmed by positive specific immunoglobulin E (IgE) (≥0.35 kU/L) (ImmunoCAP®; Phadia, Uppsala, Sweden). The allergens assessed were: house dust mites (HDM), cat and dog dander, grass and birch pollens, cow's milk proteins, egg and peanut. Specific IgE to *Alternaria alternata* and staphylococcal toxins (A, B, C, TSST (toxic shock syndrome toxin-1) were also analyzed. The Phadiatop infant test® (ImmunoCAP®; Phadia, Uppsala, Sweden) was assessed in all children and a result ≥0.35 kU/L was considered positive [E3]. We defined sensitization as positive SPT as well as positive specific IgE to ensure an accurate diagnosis.

Perennial sensitization only was defined as sensitization to HDM and/or cat or dog dander without seasonal sensitization. Sensitization to grass and/or birch pollen only without perennial sensitization was defined seasonal sensitization.

Flexible bronchoscopy

Flexible bronchoscopy was performed under local anesthesia as previously described in our centre in which more than 300 flexible bronchoscopies are routinely performed a year [E4]. Children received intra-rectal midazolam and atropine with nitrous oxyde (Entonox) during the bronchoscopy. Broncho-alveolar lavage (BAL) was obtained as per European Respiratory Society guidelines [E5] and processed for cytology by the hospital cytopathology department. Total cell count was expressed as an absolute rate. Eosinophil and neutrophil cells were expressed in percentage of the total cell count.

Inflammatory markers

Nonspecific inflammatory markers were measured in peripheral blood. These included neutrophil counts expressed in absolute rate per mm^3 (cell counting by automated Sysmex®; Roche Diagnostics, Roissy, France), serum IgG, IgA and IgM levels (g/L) using the immunoturbidimetry technique (PLC Modular®; Roche Diagnostics, Meylan, France) and percentage of eosinophil and neutrophil cells in BAL.

Variable reduction and data transformation

The initial dataset provided almost 100 variables selected from a large spectrum of routine assessments of asthmatic children. The following *a priori* strategies were used to reduce the number of variables before performing the cluster analysis. Selecting variables reflecting major physiological parameters reduced the number of variables that were clinically

redundant. Spectrums of response variables were transformed into "composite variables", e.g. perennial sensitization only was defined as sensitization to HDM and/or cat or dog dander without seasonal sensitization or sensitization to grass and/or birch pollen only without perennial sensitization defined as seasonal sensitization.

In the end, 34 variables were included in the cluster analysis: (1) age at the time of exploration; (2) age of asthma onset; (3) >75th percentile BMI; (4) gender; (5) parents' socio-professional categories; (6) ethnic group; (7) maternal history of asthma; (8) paternal history of asthma; (9) history of pneumonia; (10) history of GERD; (11) active allergic rhinitis; (12) active atopic dermatitis; (13) IgE-mediated food allergy; (14) more than two hospitalizations for severe exacerbation during the previous year; (15) wheezing triggers; (16) annual number of antibiotics for pneumonia; (17) asthma severity; (18) perennial sensitization only; (19) seasonal sensitization only; (20) both sensitization to perennial and seasonal allergens; (21) sensitization to egg and/or cow's milk proteins; (22) peanut sensitization; (23) *Alternaria alternata* sensitization; (24) staphylococcal toxin sensitization; (25) Phadiatop Infant test® $\geq$0,35KU/l; (26) absolute value of blood neutrophil; (27) absolute value of blood eosinophil; (28) serum total IgE (kU/L); (29) IgG level (g/L); (30) IgA level (g/L); (31) IgM level (g/L); (32) BAL neutrophil percentage; (33) BAL eosinophil percentage; (34) BAL microbiological cultures.

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Table E1: Environmental exposure associated with cluster phenotypes

	Entire population n=350	**Cluster 1** Neutrophilic steroid-refractory recurrent wheeze phenotype **n= 138**	**Cluster 2** Severe recurrent wheeze with sensitization to a single aeroallergen **n= 104**	**Cluster 3** Eosinophilic steroid-refractory asthma phenotype **n= 108**	**p value** *
Tobacco smoke exposure n (%)	118 (34)	53 (38)	31 (30)	34 (31)	NS
Molds n (%)	76 (22)	27 (19)	24 (23)	25 (23)	NS
Cockroaches n (%)	23 (7)	6 (4)	10 (10)	7 (6)	NS
Daycare attendance n (%)	122 (35)	52 (38)	**43 (42)**	27 (25)	0.03
Overcrowding n (%)	31 (9)	8 (6)	12 (12)	11 (10)	NS

NS, Nonsignificant

Boldface values indicate statistical significance

*χ^2 test when conditions allowed or Fisher's exact test otherwise

Table E2: Staphylococcal toxin sensitization according to cluster analysis

	Entire population n=350	**Cluster 1** Neutrophilic steroid-refractory recurrent wheeze phenotype **n= 138**	**Cluster 2** Severe recurrent wheeze with sensitization to a single aeroallergen **n= 104**	**Cluster 3** Eosinophilic steroid-refractory asthma phenotype **n= 108**	**p value** *
Staphylococcal toxin sensitization n (%)					<0.001
Absence	249 (79)	109 (92)	94 (96)	**46 (48)**	
A toxin	23 (7)	6 (5)	2 (2)	**15 (16)**	
B toxin	20 (6)	2 (2)	2 (2)	**16 (17)**	
C toxin	15 (5)	0 (0)	0 (0)	**15 (16)**	
TSST toxin	5 (2)	1 (1)	0 (0)	**4 (4)**	

TSST, toxic shock syndrome toxin-1
Boldface values indicate statistical significance
All IgE values were positive if ≥ 0.35kU/L.
*χ^2 test when conditions allowed or Fisher's exact test otherwise