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Sensitization to dust mite defines different phenotypes of asthma: a multicenter study

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Sensitization to dust mite defines different phenotypes of asthma: a multicenter study

Pediatr Allergy Immunol

ABSTRACT

Background: Indoor allergens are risk factors for asthma: thus, the characterization of indoor air quality is important for studying environment-health relationships in children. In particular, *Dermatophagoides pteronyssinus* is the dominant allergen for asthma. We cross-sectionally investigated the relationships among respiratory symptoms and function, airway inflammation, allergen sensitization and indoor allergen concentration.

Methods: One hundred thirty two children aging 10-14 years and living in a Southern Mediterranean area were evaluated by parental questionnaires. Spirometry, exhaled nitric oxide (FeNO), skin prick tests, total and specific serum IgE analyses were performed along with the

evaluation of home dust samples for the content in Der p 1 allergen. Three clusters were created on the basis of the presence/absence of wheeze in the last 12 months (Wh12m) and Der p 1 specific IgE level.

Results: Cluster 1 (Wh12m+/high Der p 1 IgE) presented higher FeNO and poorer pulmonary function (lower FEV₁ and FEF_{25-75%}), while its symptom score was not different from Cluster 2 (Wh12m+/low Der p 1 IgE). Cluster 3 (Wh12m-/low IgE) showed the lowest FeNO values and pulmonary function similar to Cluster 2. Within Cluster 1, both Der p 1 specific IgE and FeNO were positively correlated to dust Der p 1.

Conclusions: Similar asthma phenotypes may occur in children despite differences in their atopic state. In atopic children, sensitizing allergens in the indoor environment may increase airway inflammation worsening pulmonary function. Moreover, environmental exposures may contribute to the development of asthma-like symptoms also in absence of atopic sensitization, thus contributing to asthma overdiagnosis.

Key words: Asthma risk factors, Cluster analysis, Exhaled nitric oxide, Indoor allergens, Spirometry

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INTRODUCTION

In the last decades various research programs on health effects of indoor air quality have been developed worldwide, regarding both school (1) and domestic (2) indoor environments in an attempt to find associations between environmental pollutants and respiratory diseases. In particular, indoor environment has been recognized as potential risk factor for childhood asthma (3). Being that asthma is a heterogeneous disease, the identification of specific phenotypes may increase precision for the diagnosis and treatment of the disease: Global Initiative for Asthma (GINA) guidelines defined asthma phenotypes as some recognizable clusters of demographic, clinical, and/or pathophysiological characteristics (4).

Children belong to the category of “vulnerable individuals” spending much of their time indoors (5). Thus, the characterization of the indoor environment is important in the study of its relationship with health. Observational studies already addressed this point showing an inconsistent association between symptoms of asthma and, for instance, allergen exposure (6).

Exhaled nitric oxide (FeNO) is produced by airway epithelial cells in response to inflammatory cytokines and it has been suggested as an useful non invasive marker available for assessing lung inflammation derived from a specific IgE-dependent mechanism which is typically involved in eosinophilic inflammation (7). However, some discrepancies exist between different studies dealing with the association between FeNO levels and the quality of life of children with allergic rhinitis (8, 9).

The RESPIRA Project was conducted on the Gela surroundings (southern Italy) and in Malta, in the southern Mediterranean area where the presence of high concentration of environmental contaminants has been reported in both Sicilian (10) and Maltese (11) areas. Aimed at investigating the relationships among individual atopic sensitization, domestic exposure to indoor allergens, and respiratory health and function, children living in those areas underwent clinical evaluation and their dwellings studied. A cluster analysis method was adopted for studying subjects within

homogeneous subsamples. Therefore, we evaluated the possible relationships among clusters and indoor allergens.

METHODS

Study design

Between March and December 2012, in the context of *RESPIRA* project, within the Cross-Border Program Italy-Malta 2007-2013, a survey was performed in Sicily and Malta, two islands in the Mediterranean Sea. In Sicily, 1,325 schoolchildren were randomly selected in the Gela Health District, 1,075 in Malta (On-line Repository Figure 1).

The study was approved by the local Ethical Committees. All parents of the children signed an informed consent. The respect of individual privacy concerning clinical data was granted.

Questionnaires

Parents of investigated children filled out a questionnaire regarding respiratory symptoms and personal information: 2,053 parental questionnaires were available (85.5% of all selected children). Based on questionnaire responses (see Questionnaires section in the On-line repository), a sample of 173 children presenting asthma symptoms in the last 12 months (“Cases”) was identified (111 in Sicily and 62 in Malta). Among children not presenting any history of asthma, 169 “Controls” (111 in Sicily and 58 in Malta) were randomly selected.

An individual symptom score (0-8) was created from a pool of 8 questionnaire items, 7 concerning asthma related symptoms, one on eczema (Methods section and Table 1 in the On-line repository). In addition, aimed at evaluating the severity of asthma symptoms, a question relevant to the use of asthma medication was introduced.

Pulmonary function and exhaled nitric oxide measurements

By means of a phone contact, children's parents were requested to referring their children to the local clinics where the subjects were submitted to clinical tests by experienced physicians and technicians. Pulmonary function tests were performed through portable spirometers and measured according to ATS/ERS guidelines (12). Predicted values were those from Quanjer et al. (13). Exhaled nitric oxide was also measured according to the American Thoracic Society and European Respiratory Society guidelines (14).

Skin Prick Tests

Skin prick tests included *Dermatophagoides*, *Alternaria*, *Parietaria*, Graminaceae, Dog, *Olea*, *Blattella germanica*, Cat extracts plus positive and negative controls, according to EAACI recommendations (15). Atopic index (AI) was computed for each subject as the sum of mean wheal diameters of positive reactions (16) (see On-line repository for details).

Sampling sites and blood assays

A total of 156 homes were analyzed for Der p 1 level in dust samples (75 in Sicily and 81 in Malta, see also On-line repository). Total and specific IgE were quantified as described in On-line repository.

Statistical analyses

SPSS two-step cluster analysis – an exploratory tool aimed at revealing natural groupings within a dataset – was used for creating different subjects' clusters on the basis of the presence/absence of wheeze in the last 12 months (Wh12m) and Der p 1 specific IgE level. The algorithm consists of a pre-cluster step that pre-clusters cases into sub-clusters and subsequently a cluster step that hierarchically clusters the sub-clusters into the desired number of clusters. Because

SPSS assumes that continuous variables are normally distributed, the natural log of Der p 1 specific IgE was used.

Categorical variables were compared by means of χ^2 test, continuous variables by one-way ANOVA and Mann-Whitney U-test or Kruskal-Wallis test for normally and not normally distributed variables respectively. Correlation between log transformed variables was tested by linear regression analysis. Analysis of Covariance was used for evaluating differences among slopes. P values <0.05 were considered significant.

Detailed method description is provided in On-line repository.

RESULTS

Complete information was available on 125 children aged 10-14 years (67 Cases [36 in Italy and 31 in Malta] and 58 Controls [32 and 26, respectively]) and their homes. Table 1 shows descriptive data of the sample. Allergic sensitization and serum total and Der p 1 specific IgE level were significantly different between Cases and Controls, higher among Cases. None of the children was an active smoker. Between Sicily and Malta no significant difference was found in both continuous and categorical variables (data not shown).

Atopic state

As regards Skin Prick Tests, *Dermatophagoides pteronyssinus* yielded 32% positivity in the overall sample (Cases 45%, Controls 17%, $p=0.001$), being by far the most frequent allergen (On-line repository, Table 1). The median values of serum Der p 1 specific IgE were significantly higher among Cases than among Controls ($p=0.007$, Table 1 and On-line repository, Table 2). Nevertheless, lower values of Der p 1 specific IgE levels in Cases and higher ones in Controls largely overlapped.

Clusters

Two-step cluster analysis was used for creating clusters founded on the questionnaire-based presence of Wh12m and Der p 1 specific IgE level. Three clusters were produced (Table 2): Cluster 1 was Wh12m+, with high Der p 1 specific IgE (median and IQR 15.01 [8.41-43.22], IU/ml); Cluster 2 was Wh12m+ and low Der p 1 specific IgE (0.22 [0.19-0.26]); Cluster 3 was Wh12m- and low Der p 1 specific IgE (0.23 [0.19-0.27]). A value of 2.70 IU/ml of Der p 1 IgE separated Cluster 1 from Cluster 2, higher than that of 0.34 IU/ml regarded as positive following the manufacturer's recommendation. Sensitization for *Dermatophagoides pteronyssinus* was strongly more frequent in Cluster 1 with respect to Clusters 2 and 3; similarly, the prevalence of sensitizations for *P. judaica*, and dog and cat dander was higher in Cluster 1 (On-line repository, Table 3). Atopic index and FeNO levels were considerably higher in Cluster 1 with respect to Clusters 2 and 3 (Table 2). Conversely, FeNO was not significantly different between Cases and Controls being 13.0 ppb (9.0-38.8) and 12.5 ppb (9.0-16.0) respectively (median and IQR, $p=0.090$, Mann-Whitney U-test). In both Clusters 1 and 2 a significant correlation was found between FeNO and Der p 1 specific IgE ($p=0.002$ and 0.034 respectively, On-line Repository Figure 2).

The symptom score (On-line repository, Table 4) was not significantly different between Clusters 1 and 2 (Figure 1), while Cluster 3 showed a lower symptom score. Similarly, the frequency of use of asthma medications was very similar in Clusters 1 and 2 (58 and 51%, respectively, On-line repository, Table 4). When each item composing the symptom score was individually assessed, rhinoconjunctivitis was the only symptom showing a large difference in frequency distribution between Cluster 1 (50.0%) and Cluster 2 (25.6%, $p=0.044$). In Cluster 1, 70.8% of subjects had a doctor diagnosis of asthma, 48.8% in Cluster 2 ($p<0.0001$, χ^2 test, Table 2). Interestingly, domestic exposure to tobacco smoke (ETS) was over twofold in Cluster 2 with respect to Cluster 1 ($p<0.018$, χ^2 test). Both maximum mid-expiratory flow (FEF_{25-75%}, % of predicted) and forced expiratory volume in one second/forced vital capacity ratio were significantly lower in Cluster 1 than Clusters 2 and 3 (Table 2 and Figure 2, panels A and B). In particular,

20.8% of subjects in Cluster 1 had an abnormal FEF_{25-75%} (i.e., <5th percentile) (13), being the same figure 4.8% in Cluster 2 and 7.0% in Cluster 3 (p=0.070, χ^2 test).

Dust Der p 1 and FeNO levels were significantly higher in Cluster 1 than in Cluster 2 (Table 2 and Figure 3, panels A and B, respectively). Within Cluster 1, Der p 1 specific IgE (Figure 4, panel A) and FeNO (Figure 4, panel B) were significantly and positively correlated to dust Der p 1 (as natural logs), while no significant relationship was obtained in Clusters 2 and 3. The Analysis of Covariance showed significant differences among slopes.

DISCUSSION

Similar asthma phenotypes – i.e., individuals with very similar symptom presentation – may occur in children living in the Mediterranean area despite relevant differences in their atopic sensitization. Sensitization to *Dermatophagoides* plays the major role in sustaining higher FeNO levels and is, in turn, positively correlated to indoor Der p 1. In sensitized individuals, a poorer pulmonary function is present.

GINA Guidelines define asthma phenotypes as some recognizable clusters of demographic, clinical, and/or pathophysiological characteristics (GINA 2014). In particular, allergic and non-allergic asthma are two of the most common phenotypes. In our sample, sensitization to *Dermatophagoides* showed both the highest prevalence among tested allergens and the largest difference between Cases and Controls. Nevertheless, when evaluated on the basis of the presence/absence of Wh12m – one of the “core” modules in the epidemiologic research on pediatric asthma (17) – a large overlap was present between the two groups: some “Cases” (Wh12m+) had a low Der p 1 specific IgE level, similar to that shown by “Controls” (Wh12m-). Moreover, FeNO, usually considered a non invasive marker of airway inflammation (7), was not significantly different between Cases and Controls (Table 1). Thus, in our sample of 132 children (74 “cases” and 58 “controls” from a general population) we looked at the combination between Wh12m and IgE level for Der p 1, the most common sensitizing allergen among atopic subjects. Thus, the

analysis of Wh12m and Der p 1 IgE level allowed us to separate three different clusters with distinct combinations of symptoms and atopy. The first two clusters, composed by subjects reporting Wh12m, are distinguished based on high/low Der p 1 IgE level. Since some controversies still exist on the clinical significance of specific IgE cut-off levels (18), we chose not to use a specific Der p 1 IgE cut-off for separating Clusters 1 and 2, leaving the selection to a SPSS two-step algorithm.

Due to selection criteria, we observed a high level of sensitization to *Dermatophagoides* in *Cluster 1*: this was also associated with both the highest AI at skin prick test and the highest total IgE level. This is not surprising being *Dermatophagoides* considered the dominant sensitization allergen for asthma (19) and recognized as one of the most common allergens for human sensitization (20). Despite clear differences in the atopic state, in both Clusters 1 and 2 we found subjects with a similar number of questionnaire-reported asthma symptoms. Only rhinoconjunctivitis showed a significant difference in frequency distribution between Clusters 1 and 2 along with an increased doctor diagnosis of asthma (Table 2): this is in agreement with a previous study on a population sample of adolescents, suggesting that rhinoconjunctivitis – associated with a higher AI – is a stronger risk factor for asthma with respect to rhinitis alone (21). Moreover, the combination within Cluster 1 of subjects' sensitization and higher exposure to sensitizing allergens possibly produced the increased frequency of doctor diagnosis of asthma (22). This is in line with the recommendation of avoiding allergen exposure in sensitized individuals present in the last update of GINA Guidelines (23).

Despite a similar symptom presentation in Clusters 1 and 2, pulmonary function was poorer in Cluster 1 than in Clusters 2 and 3 and the percentage of children with an abnormal FEF_{25-75%} (13) was four times higher in Cluster 1 than in Cluster 2. This result is in agreement with a previous population study in children showing that sensitization to mites is associated with spirometric changes in large and small airways (24). In Cluster 1 a significant correlation exists not only between Der p 1 specific IgE and dust Der p 1 allergen, but also between FeNO and dust Der p 1:

i.e., a higher presence of Der p 1 allergen in the domestic environment is able to elicit an increased response in terms of Der p 1 specific IgE along with increased airway inflammation as demonstrated by higher FeNO levels. However, this was evident only among atopic subjects: in fact, such relationships were not found in Clusters 2 and 3, composed by non atopic and asymptomatic subjects, respectively. Our data are in agreement with the findings reported from a large cohort of asthmatic children showing a significant relationship between sensitization to Der p 1 and indoor content for house dust mites (25). It is noteworthy that in Cluster 2 ETS prevalence was twofold higher than in clusters 1 and 3. In a meta-analysis by Vork et al, exposure to secondhand tobacco smoke showed a consistently positive association with asthma during childhood (26). This could explain a similar symptom score between Clusters 1 and 2, despite a large difference in atopic sensitization, as clearly demonstrated by Der p 1 specific IgE and total IgE levels, and AI.

It is noteworthy that Wh12m did not produce, *per se*, any significant effect on FeNO: in fact, FeNO level was fourfold higher in Cluster 1 (high Der p 1 specific IgE level) than in Cluster 2, characterized by a similar asthma symptom but by a largely lower Der p 1 IgE level. Moreover, FeNO was not significantly different between Clusters 2 (Wh12m+) and 3 (Wh12m-), with similar Der p 1 IgE levels. This finding is in agreement with previous data showing that in a population sample FeNO may be strongly dependent on the atopic state, being *Dermatophagoides* one of the strongest allergens in sustaining FeNO levels in children (27). In fact, we found a strong relationship between FeNO and blood Der p1 specific IgE level in Cluster 1. Similarly, in population based studies, FeNO appears to be correlated to atopy and not to pulmonary function variables (28). The higher ETS prevalence in Cluster 2 could also account for lower FeNO values: this was previously demonstrated in both adults (29) and children (30). This finding suggests that, through an increased risk of developing allergic sensitization, susceptibility and social/environmental factors may currently act in modulating the individual risk of presenting asthma symptoms (31).

Strength of this multicenter study is the standard methods used: study design, questionnaire, functional and atopic evaluation, indoor environmental data. All the operators received the same training and this allowed full comparability of data: showing similar results in different population samples, cluster analysis makes data and results immediately transferrable to clinicians for the day-by-day management of atopic children. A possible weakness may be found in the differences in sex distribution among Clusters: we observed that males were exceeding within the case group and, in turn, in Cluster 1. This is not unexpected in view of the subsampling design and the epidemiological knowledge of larger asthma and atopy prevalence among males in this age group. However, there was no significant difference in sex distribution between case and control groups.

In conclusion, through a two-step cluster analysis, this study shows that similar asthma phenotypes may occur in children living in the Mediterranean area despite relevant differences in atopic state. In atopic children, the presence of sensitizing allergens in the indoor environment may increase airway inflammation worsening pulmonary function. On the other hand, environmental exposures – such as tobacco smoke – may contribute to the development of asthma-like symptoms also in absence of atopic sensitization. This – in absence of functional evaluations – could contribute to asthma overdiagnosis.

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CONFLICT OF INTEREST

Authors have no conflict of interest to disclose

AUTHOR CONTRIBUTIONS

SR, GD VL, DB, and CZ contributed to acquisition, analysis, and interpretation of data, participating to the critical revision of the manuscript; PC, MB, SM, GLS, GC, and GV contributed to conception and design of the study, data analysis and interpretation, also participating to drafting the manuscript and to critically revising it; FC contributed to conception and design of the study, to data acquisition, analysis and interpretation, drafting and revising the article. All authors read and approved the final version of the manuscript.

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FIGURE LEGENDS

Figure 1 – Box plots relevant to the number of symptoms (as derived from individual questionnaires) separately per each cluster. Bars indicate (from the bottom to the top) 10th, 25th, 50th (median), 75th, and 90th percentiles. Values below 10th and above 90th percentiles are plotted as circles. Between group differences were evaluated (Kruskal-Wallis test)

Figure 2 – Means (+SD) of maximum mid-expiratory flow (FEF_{25-75%}, % of predicted, panel A) and forced expiratory volume in one second/forced vital capacity (FEV₁/FVC, absolute ratio, panel B), separately for each Cluster. Between group differences were evaluated (one-way ANOVA)

Figure 3 – Concentration of *Dermatophagoides pteronissynus* Der p 1 allergen in collected dwelling dust (Dust Der p 1, panel A) and exhaled nitric oxide (FeNO, panel B) levels separately per each cluster. Bars indicate (from the bottom to the top) 10th, 25th, 50th (median), 75th, and 90th percentiles. Values below 10th and above 90th percentiles are plotted as circles. Between group differences were evaluated (Kruskal-Wallis test)

Figure 4 – Correlation between natural log of Der p 1 specific IgE in blood serum (panel A) and the natural log of exhaled nitric oxide (FeNO, panel B) versus the natural log of the concentration of *Dermatophagoides pteronissynus* Der p 1 allergen in collected dwelling dust. Linear regression coefficients were computed separately per each Cluster. The Analysis of Covariance demonstrated significant difference among slopes (p=0.006 and p=0.032, respectively)

TABLES

Table 1 – Characteristics of study sample (No.=125) separately for Case and Control subjects

	Cases (No.=67)	Controls (No.=58)	p
Male/Female (No.)	38/29	28/30	0.346 [#]
Age (yrs, mean±SD)	12.1 (±1.0)	12.3 (±0.9)	0.430 [§]
BMI (kg/m ² , mean±SD)	22.8 (±5.7)	21.4 (±4.2)	0.121 [§]
Allergic sensitization* (No, %)	35 (53.8%)	20 (34.5%)	0.031 [#]
Domestic exposure to tobacco smoke (No, %)	25 (37.3%)	13 (22.4%)	0.069 [#]
FEV ₁ (% of predicted, mean±SD)	102.5 (±15.2)	99.2 (±13.5)	0.211 [§]
FVC (% of predicted, mean±SD)	104.5 (±16.6)	99.1 (±15.0)	0.062 [§]
FEF _{25-75%} (% of predicted, mean±SD)	96.4 (±22.9)	97.5 (±21.5)	0.768 [§]
FEV ₁ /FVC (absolute %, mean±SD)	0.85 (±0.06)	0.88 (±0.06)	0.057 [§]
FeNO (ppb, median and IQ range)	13.0 (9.0-38.8)	12.5 (9.0-16.0)	0.090 [‡]
Total IgE (IU/ml, median and IQ range)	64.8 (21.4-223.0)	41.8 (7.2-92.0)	0.027 [‡]
Atopic Index (mm, median and IQ range)	3.5 (0.0-11.1)	0.0 (0.0-5.5)	0.066 [‡]
Specific Der p 1 IgE (IU/ml, median and IQ range)	0.27 (0.21-9.00)	0.23 (0.19-0.27)	0.007 [‡]

*At least one positive skin prick test

[#] χ^2 test; [§]one-way ANOVA; [‡]Mann-Whitney U-test

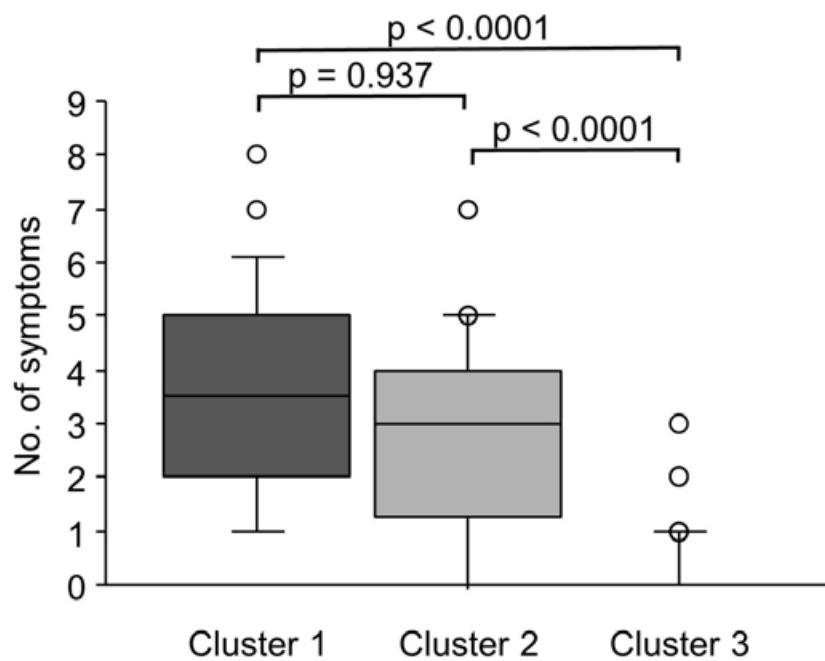
Table 2 – Characteristics of clusters created on the basis of the presence of wheeze in the last 12 months and specific blood IgE for Der p 1 (IgE Der p 1) level (IQ range: interquartile range).

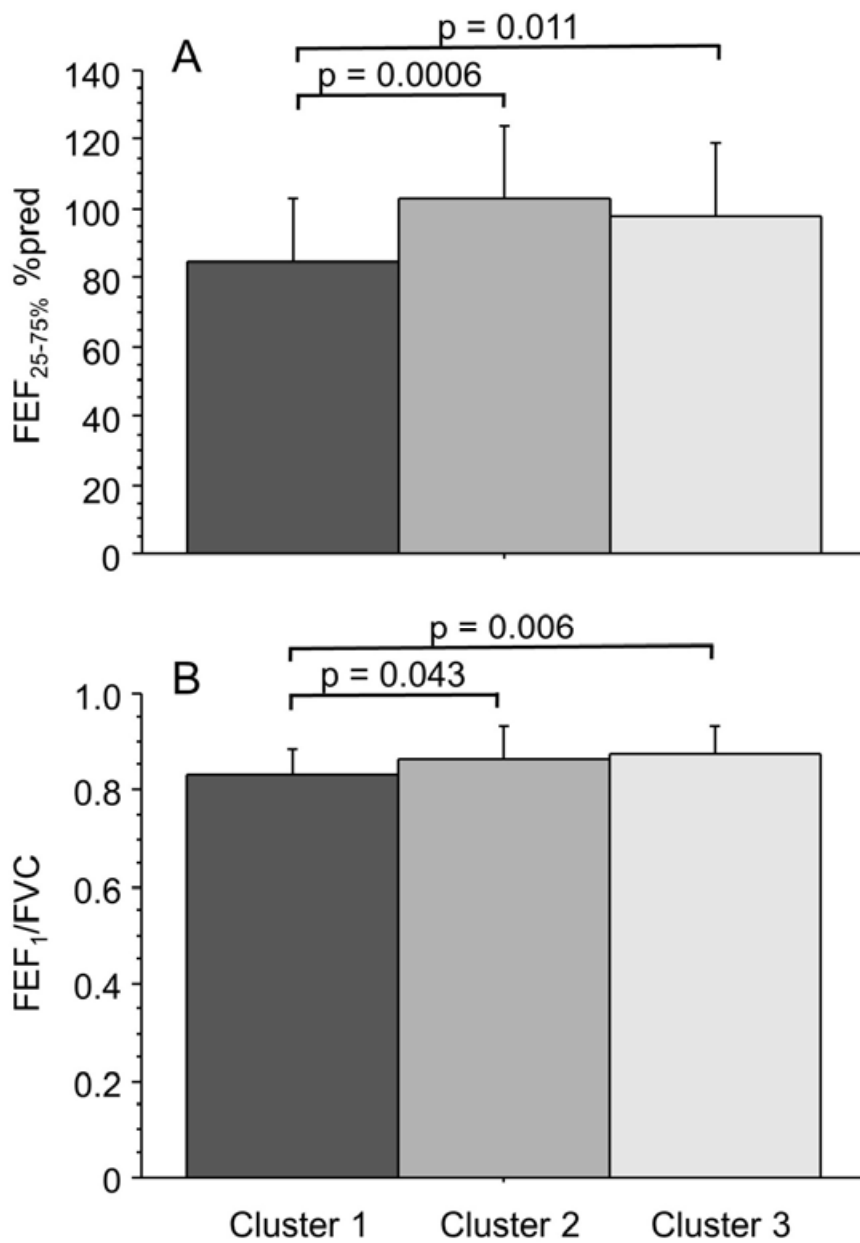
The variables on a grey background are those used for cluster definition.

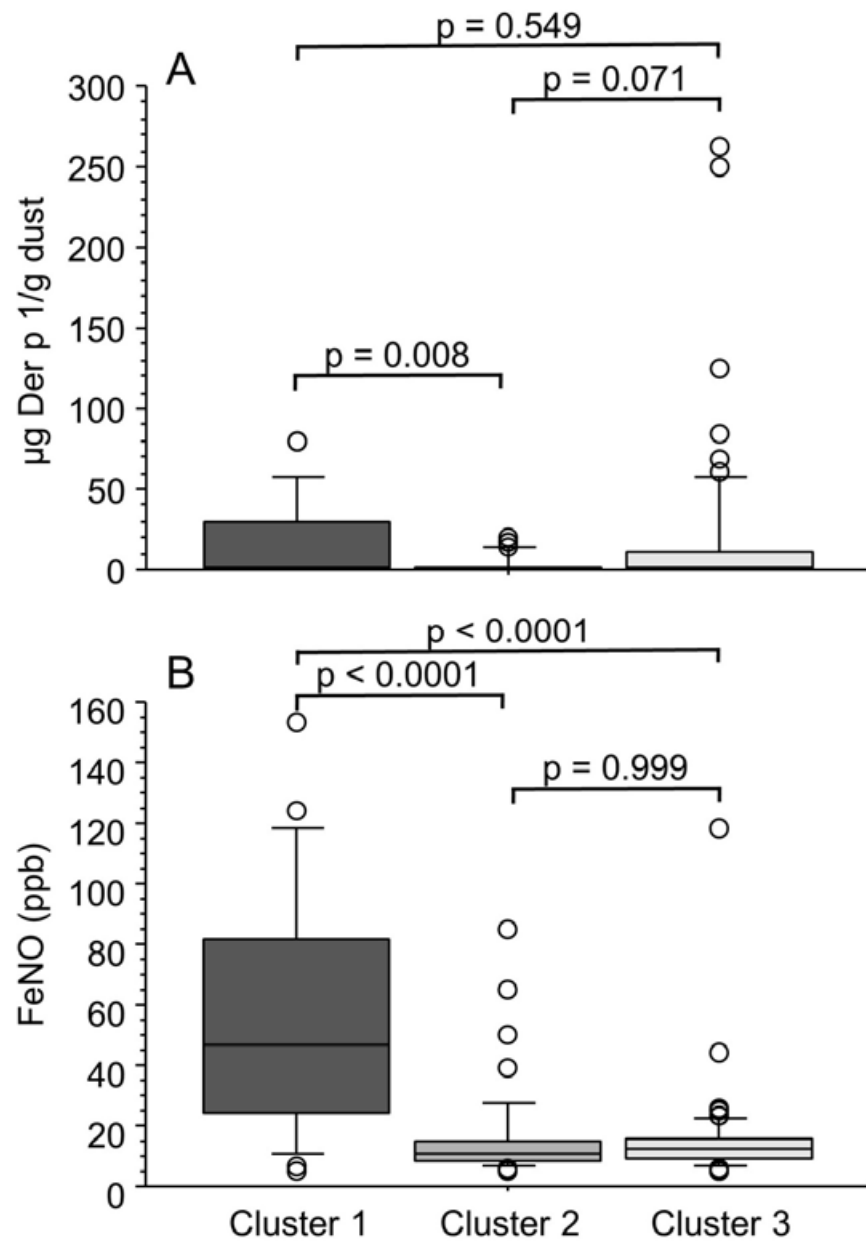
	Cluster 1 (No.=24)	Cluster 2 (No.=43)	Cluster 3 (No.=58)	p value
Wheeze in the last 12 months	Yes	Yes	No	
High specific Der p 1 IgE	Yes	No	No	
Specific Der p 1 IgE (IU/ml, median and IQ range)	15.0 (8.4-43.2)	0.2 (0.2-0.3)	0.2 (0.2-0.3)	N.C.
Male/Female (No.)	15/9	23/20	28/30	0.499 [†]
Atopic index (mm, median and IQ range)	11.5 (6.8-15.8)	0.0 (0.0-3.5)	0.00 (0.0-5.5)	<0.0001 [§]
Total IgE (IU/ml, median and IQ range)	270.2 (127.1-509.8)	30.1 (8.3-74.4)	41.8 (7.2-92.0)	<0.0001 [§]
BMI (kg/m ² , mean±SD)	22.3±5.6	23.1±5.8	21.4±4.2	0.245 [†]
Dust Der p 1 (µg allergen/g dust, median and IQ range)	2.1 (0.8-30.1)	0.5 (0.2-2.3)	1.9 (0.0-10.2)	0.007 [§]
FeNO (ppb, median and IQ range)	46.5 (24.0-81.5) [§]	11.0 (8.0-15.0) [§]	12.5 (9.0-16.0)	<0.0001 [§]
Doctor diagnosed asthma (No, %)	17 (70.8)	21 (48.8)	1 (1.7)	<0.0001 [†]
Domestic exposure to tobacco smoke (No, %)	5 (20.8)	20 (46.5)	13 (22.4)	0.018 [†]
FEV ₁ /FVC (mean ± SD)	0.83±0.05	0.87±0.07	0.88±0.06	0.021 [^]
FEF _{25-75%} (% of predicted, mean and SD)	84.5±18.1	103.2±21.1	97.5±21.5	0.003 [^]

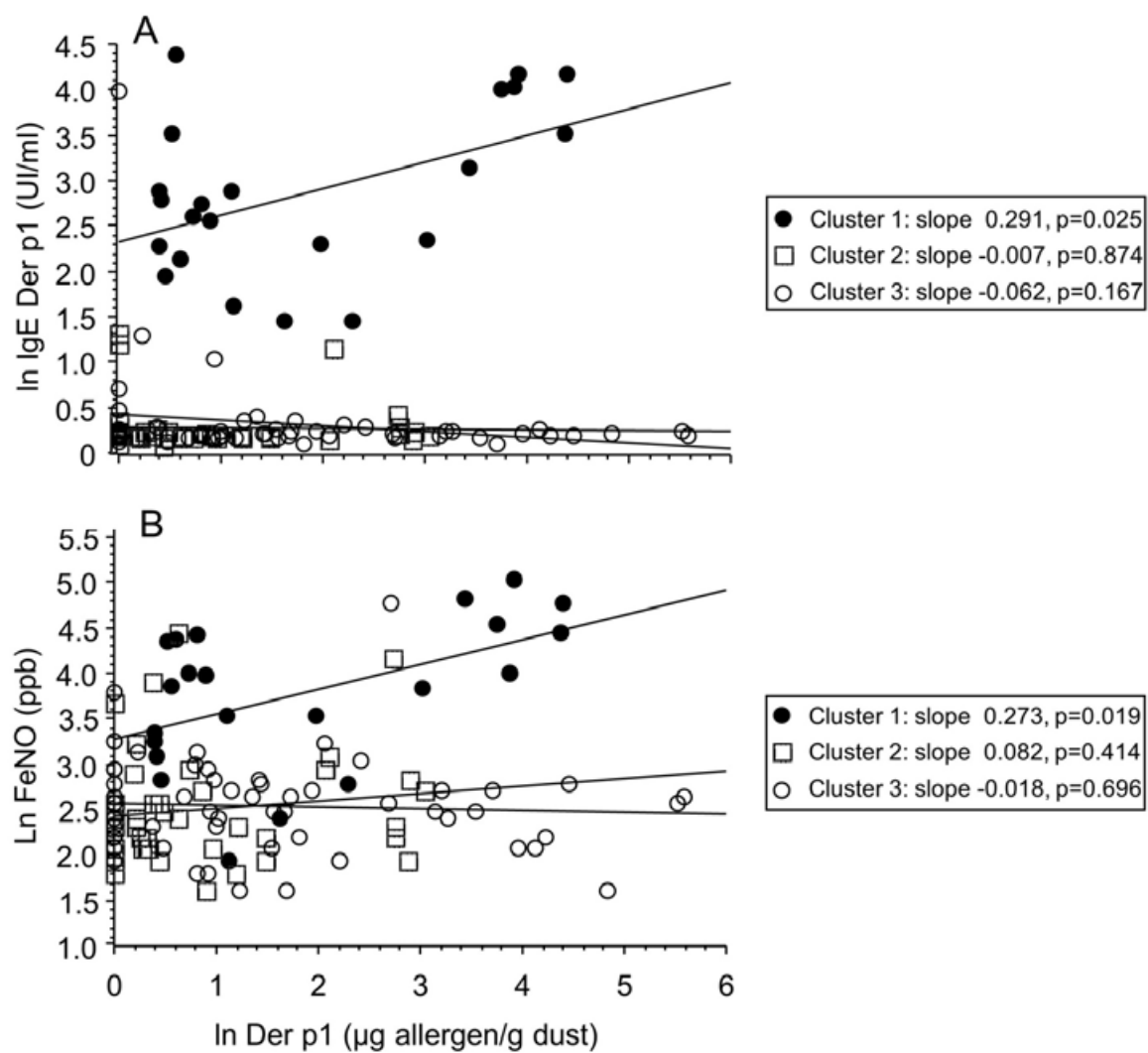
[†] χ^2 test; [§] Kruskal-Wallis test; [†]one-way ANOVA; [^]one-way ANOVA

N.C.: the p value relevant to Specific blood Der p 1 IgE was not computed being the variable used for cluster definition



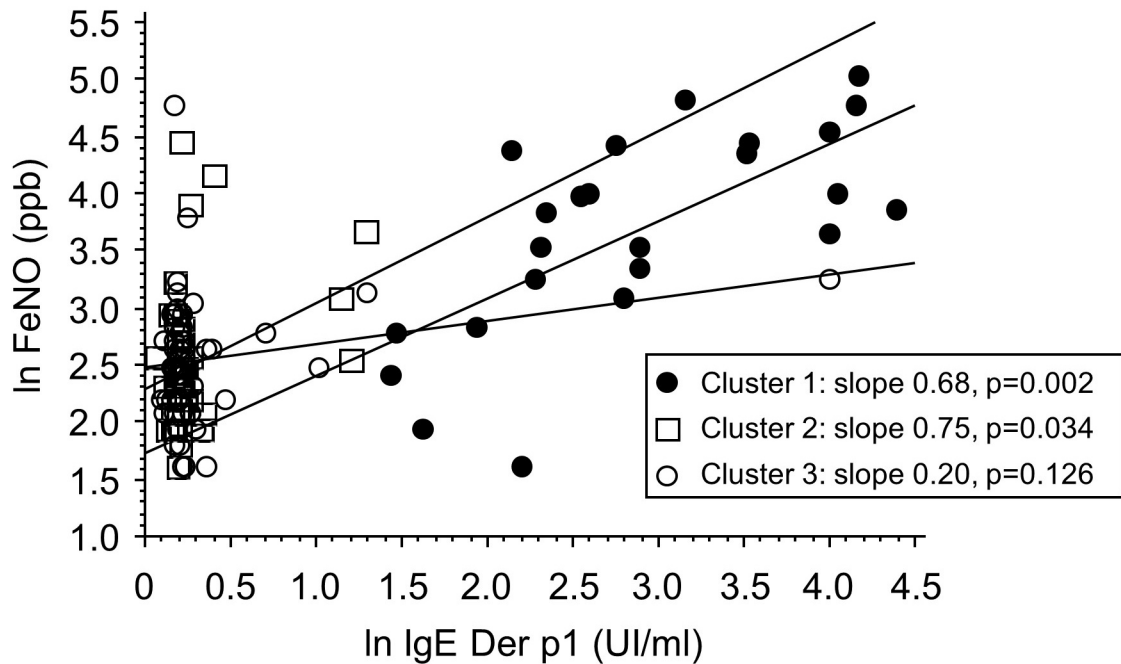








On-line Repository Figure 1



On-line Repository Figure 2

Appendix S1. On-line Repository

METHODS

Study design

Between March and December 2012, in the context of *RESPIRA* project developed within the Cross-Border Cooperation Program Italy-Malta 2007-2013, an epidemiological survey was performed in Sicily and Malta, two islands in the southern Mediterranean area. For the Sicilian side, a total of 1,325 schoolchildren were randomly selected from all the 12 secondary schools - first degree - of Gela (77,000 inhabitants/660 evaluated subjects), Niscemi (26,400/355), Mazzarino (11,800/186), and Butera (4,900/124). For the Maltese side (416,000 inhabitants), a total of 1,075 schoolchildren were selected from six Maltese secondary schools - first degree (On-line Repository Figure 1).

The study was approved by the local Ethical Committees. All parents of the invited children signed a written informed consent. According to the Italian and Maltese laws, the respect of individual privacy concerning clinical data was granted.

Questionnaires

Parents of all investigated children fulfilled a respiratory questionnaire regarding past and current respiratory allergic symptoms and other personal information. Moreover, domestic exposure to tobacco smoke (ETS) was investigated by the question “*Is your child exposed to tobacco smoke in the dwelling?: Yes, daily / Yes often (1-4 times/week) / Yes, sometimes (1-3 times/month) / No, never*”. Subjects were considered non exposed if the answer was “*No, never*”: 2,053 parental questionnaires were available (85.5% of all the selected children). On the basis of questionnaire responses, all the 173 children with asthmatic respiratory symptoms (“Cases”, 8.4% of 2,053) were identified (111 in Sicily and 62 in Malta) when providing a positive answer to the following question: “*Has your child had wheezing or whistling in the chest in the last 12 months*”; plus a positive answer to one of the following two: “*In the past 12 months, has your child's chest sounded wheezy during or after exercise?*”; “*In the past 12 months, has your child had a dry cough at night,*

apart from a cough associated with a cold or chest infection?”. By means of a computer-based procedure, 169 control children (“Controls”, 111 in Sicily and 58 in Malta) were randomly selected among those with a negative answer to the above mentioned questions along with a negative response to the following: *“Has your child ever had wheezing or whistling in the chest at any time in the past?”*.

Other information about additional symptoms (including rhinitis) and domestic smoking habits were investigated. Domestic exposure to tobacco smoke (ETS) was investigated by the question *“Is your child exposed to tobacco smoke in the dwelling?: Yes, daily / Yes often (1-4 times/week) / Yes, sometimes (1-3 times/month) / No, never”*. Subjects were considered non exposed if the answer was *“No, never”*. Mould/dampness exposure was evaluated using the question: *“Is there dampness or visible mould growth in your child’s bedroom?”*. On the basis of the available resources, for the purposes of the present study we randomly selected 140 subjects: 41 Cases and 38 Controls on the Sicilian side and 33 Cases and 28 Controls on the Maltese one.

Symptom score.

For each subject a Symptom Score was created (0-8) by means of the following questions (frequency distribution of answers to questionnaire questions is provided in On-line Repository Table 1):

- *“Has your child had wheezing or whistling in the chest in the last 12 months?”* for exercise wheezing in the last 12 months;
- *“In the past 12 months, has your child had a dry cough at night, apart from a cough associated with a cold or chest infection?”* for nocturnal cough;
- *“In the past 12 months, did your child use any drugs (pills, sprays, nebulizers or any other remedies) for asthma?”* for use of asthma medications;

- *“Does your child have cough on most days (4 or more days for week) apart from common colds?”* subjects were considered positive for chronic cough if they answered at least 1 month per year;
- *“Does your child have phlegm on most days (4 or more days for week) apart from common colds?”* subjects were considered positive for chronic phlegm if they answered at least 1 month per year;
- *“Have your child ever had a problem with sneezing, or runny, or blocked nose apart from common cold or flu in the past 12 months?”* for rhinitis in the last 12 months;
- Rhinoconjunctivitis was defined for a positive response to the previous question plus to *“In the past 12 months, has this nose problem been accompanied by itchy - watery eyes?”*;
- *“Has your child ever had eczema?”* for Eczema.

In addition, aimed at evaluating the severity of asthma symptoms, a question relevant to the use of asthma medication was introduced:

- *“In the past 12 months, did your child use any drugs (pills, sprays, nebulizers or any other remedies) for asthma?”*.

Pulmonary function and exhaled nitric oxide measurements

Children’s parents were requested by means of a phone contact to referring their children to the local clinics where the subjects were submitted to clinical tests by experienced physicians and technicians: pulmonary function tests were performed through portable spirometers (MicroLoop, Micro Medical, Chatham Maritime, Kent, UK). Forced expiratory volume in one second (FEV₁), forced vital capacity (FVC) and maximum mid-expiratory flow (FEF_{25-75%}) were measured according to ATS/ERS guidelines (1): the best FVC and FEV₁ values were retained and FEF_{25-75%} was selected from the manoeuvre with the largest sum of FEV₁ and FVC. Spirometric predicted values were those from Quanjer et al. (2). For each subject, FEV₁/FVC and FEF_{25-75%}/FVC ratios were also computed.

Exhaled nitric oxide measurements were performed according to the American Thoracic Society and European Respiratory Society guidelines (3) using NIOX MINO instruments (Aerocrine AB, Sweden).

Skin Prick Tests

Subjects were submitted to skin prick tests, performed according to EAACI recommendations (4), with a standard panel including *Dermatophagoides*, *Alternaria*, *Parietaria*, Graminaceae, Dog, *Olea*, *Blattella germanica*, Cat, plus a positive (histamine 1%) and a negative (saline) control (ALK-Abelló, Lainate-Milan, Italy). Allergic sensitization was defined as at least one positive skin prick test, i.e., when the mean wheal diameter (computed as the maximum diameter plus its orthogonal divided by 2) was 3 mm or greater, after having subtracted the wheal diameter of the negative control reaction. Frequency distribution of positive responses to tested allergens is provided in On-line Repository Table 1, separately for Case and Control subjects. Atopic index (AI) was computed for each subject as the sum of mean wheal diameters of positive reactions.

Sampling sites and blood essays

A total of 156 homes were analyzed for biological indoor allergens. Dust samples were collected from 75 homes in Sicily (41 cases and 34 controls) and 81 homes in Malta (42 cases and 39 controls). Only samples containing > 20mg of fine dust were considered for subsequent analysis of Der p 1 allergen with monoclonal antibodies.

Dust extraction and ELISA assay

Samples were obtained using the vacuum-cleaner Hoover Free-Space Evo 2000W equipped with a dust collector device (DustreamTM collector and filters, Indoor Biotechnologies, Cardiff, UK). In houses samples were collected in the living-room, from six areas of the floor (one square meter each, carpet included) and six areas upon the floor (sofa, pillows, chairs, tables, etc.). Immediately after the collection, the filters (containing the dust) were closed into a pre-labeled and sterile zip-lock bags, than stored at -20°C until use. Samples were extracted according to the Manufacturer's protocol (Indoor Biotechnologies, Cardiff, UK) and centrifuged for 20 minutes at 2500 rpm at 4°C.

The supernatants were divided in aliquots (200 µl each) and stored at -20°C until analysis. Dust samples were analyzed for Der p 1 allergen with monoclonal antibodies-based ELISAs using a quantitative ELISA kit (Indoor Biotechnologies, Cardiff, UK). The results were reported in ng of allergen/ml and then converted in µg of allergen/g of dust.

Total and specific IgE determination

The assay system utilizes one monoclonal anti-IgE antibody for solid phase (microtiter wells) immobilization and goat anti-IgE antibody in the antibody-enzyme (horseradish peroxidase) conjugate solution. The final colorimetric reaction is stopped with the addition of Stop Solution, and the color is changed from blue to yellow and measured spectrophotometrically at 450 nm. The total IgE concentration is directly proportional to the color intensity of the test sample and is expressed in IU/ml (cut-off >188 IU/ml). Patients specific IgE were analyzed using the RIDASCREEN[®] Spec. IgE kit (R-Biopharm AG, Germany). The allergens are attached to the cellulose discs. In order to carry out the test, the allergen discs are placed in the wells of a microwell plate. Patient sera, standard sera and negative and positive control are pipetted onto the allergen discs and incubated at 37 °C. Non-attached material is removed by washing. Subsequently, a monoclonal anti-human IgE antibody conjugated with alkaline phosphatase is added. Any non-attached conjugate is removed by washing. Substrate is then added and dephosphorylated by the conjugated enzyme into a yellow product. The intensity of the yellow colour is proportional to the quantity of allergen-specific antibodies in the serum. Photometry is carried out at 405 nm using a reference wavelength of 620 nm. The specific IgE concentration is expressed in IU/ml. Blood specific IgE levels for all tested allergens are provided in On-line Repository Table 3, separately for Cases and Controls.

Statistical analyses

SPSS two-step cluster analysis – an exploratory tool aimed at revealing natural groupings within a dataset – was used for creating different subjects' clusters on the basis of the presence/absence of wheeze in the last 12 months (Wh 12m) and Der p 1 specific IgE level. SPSS

two-step algorithm consists of a pre-cluster step that pre-clusters cases into sub-clusters and subsequently a cluster step (by hierarchical methods) that clusters the sub-clusters resulting from pre-cluster step into the desired number of clusters. Because SPSS assumes that continuous variables are normally distributed, the natural log of Der p 1 specific IgE was used.

Comparisons of categorical variables were performed by means of χ^2 test. Continuous variables were compared by one-way ANOVA and Mann-Whitney U-test or Kruskal-Wallis test for normally and not normally distributed variables respectively. Correlation between variables was tested by linear regression analysis after transformation into natural logs. Analysis of Covariance was used for evaluating differences among slopes. The analyses were carried out using Statistical Package for Social Sciences (SPSS Inc., Chicago, IL) for Windows version 20.0. P values <0.05 were considered significant.

ON-LINE REPOSITORY REFERENCES

1. Miller MR, Hankinson J, Brusasco V, Burgos F, Casaburi R, Coates A, et al. Standardisation of spirometry. *Eur Respir J* 2005; 26: 319-38.
2. Quanjer PH, Stanojevic S, Cole TJ, Baur X, Hall GL, Culver BH, et al. Multi-ethnic reference values for spirometry for the 3-95-yr age range: the global lung function 2012 equations. *Eur Respir J* 2012; 40: 1324-43.
3. American Thoracic S, European Respiratory S. ATS/ERS recommendations for standardized procedures for the online and offline measurement of exhaled lower respiratory nitric oxide and nasal nitric oxide, 2005. *Am J Respir Crit Care Med* 2005; 171: 912-30.
4. Position paper: Allergen standardization and skin tests. The European Academy of Allergology and Clinical Immunology. *Allergy* 1993; 48(14 Suppl): 48-82.

ON-LINE REPOSITORY FIGURE LEGENDS

On-line Repository Figure 1 – Geographical localization of the investigated areas

On-line Repository Figure 2 – Correlation between natural log of exhaled nitric oxide (FeNO) and natural log of Der p 1 specific IgE in blood serum. Linear regression coefficients were computed separately per each Cluster.

ON-LINE REPOSITORY TABLES

On-line Repository Table 1 - Frequency distribution of positive responses to tested allergens in skin prick tests, separately for Case (No.=67) and Control subjects (No.=58)

Allergen	Cases (%)	Controls (%)	p value (χ^2 test)
<i>Dermatophagoides pteronyssinus</i>	44.6	17.2	0.0011
<i>Blattella germanica</i>	4.6	5.2	0.886
<i>Olea europaea</i>	13.8	13.8	0.993
Grass pollen	4.6	10.3	0.223
<i>Parietaria judaica</i>	13.8	1.7	0.014
<i>Alternaria alternata</i>	6.2	8.6	0.600
Cat	12.3	3.4	0.073
Dog	4.6	3.4	0.744

On-line Repository Table 2 - Blood specific IgE levels for all tested allergens, separately for Cases and Controls (medians and interquartile ranges)

Allergen	Cases (No.=67)	Controls (No.=58)	p value (Mann-Whitney U-test)
<i>Olea europaea</i>	0.24 (0.21-0.27)	0.24 (0.21-0.26)	0.660
Goldenrod <i>Solidago virgaurea</i>	0.22 (0.19-0.25)	0.22 (0.18-0.25)	0.872
<i>Parietaria judaica</i>	0.25 (0.20-0.28)	0.23 (0.19-0.24)	0.012
Cat	0.25 (0.22-0.32)	0.24 (0.21-0.31)	0.306
Dog	0.24 (0.20-0.28)	0.24 (0.22-0.28)	0.733
<i>Cladosporium herbarum</i>	0.21 (0.18-0.22)	0.21 (0.18-0.22)	0.534
<i>Alternaria alternata</i>	0.21 (0.19-0.25)	0.21 (0.19-0.24)	0.538
<i>Dermatophagoides pteronyssinus</i>	0.27 (0.21-9.00)	0.23 (0.19-0.27)	0.0065

On-line Repository Table 3 - Frequency distribution (percentage) of positive skin prick test for evaluated allergens within each Cluster.

	Cluster 1 (No.=24)	Cluster 2 (No.=43)	Cluster 3 (No.=58)	p*
<i>Dermatophagoides pteronyssinus</i>	95.7	16.7	17.2	<0.0001
<i>Blattella germanica</i>	8.7	2.4	5.2	0.523
<i>Olea europaea</i>	26.1	7.1	13.8	0.107
Grass pollen	8.7	2.4	10.3	0.308
<i>Parietaria judaica</i>	26.1	7.1	1.7	0.001
<i>Alternaria alternata</i>	8.7	4.8	8.6	0.736
Cat dander	30.4	2.4	3.4	<0.0001
Dog dander	13.0	0.0	3.4	0.037

Cluster 1: Wheeze in the last 12 months (Wh 12m) and high IgE Der p 1

Cluster 2: Wh12m and low IgE Der p 1

Cluster 3: absence of Wh12m and low IgE Der p 1

* χ^2 test

On-line Repository Table 4 - Frequency distribution of answers to questionnaire questions within each Cluster (% [and absolute count])

Question	Cluster 1 (No.=24)	Cluster 2 (No.=43)	Cluster 3 (No.=58)	p (χ^2 test)
Exercise wheezing	52.2% (12)	53.8% (21)	0% (0)	<0.0001
Nocturnal cough	50.0% (12)	53.7% (22)	9.1% (5)	<0.0001
Use of asthma medications	58.3% (14)	51.2% (22)	1.7% (1)	<0.0001
Chronic cough	29.2% (7)	25.6% (11)	0% (0)	0.0003
Chronic phlegm	25.0% (6)	20.9% (9)	3.4% (2)	0.0052
Rhinitis in the last 12 months	70.8% (17)	48.8% (21)	3.4% (2)	<0.0001
Rhinoconjunctivitis	50.0% (12)	25.6% (11)	1.7% (1)	<0.0001
Eczema	25.0% (6)	16.3% (7)	3.4% (2)	0.010