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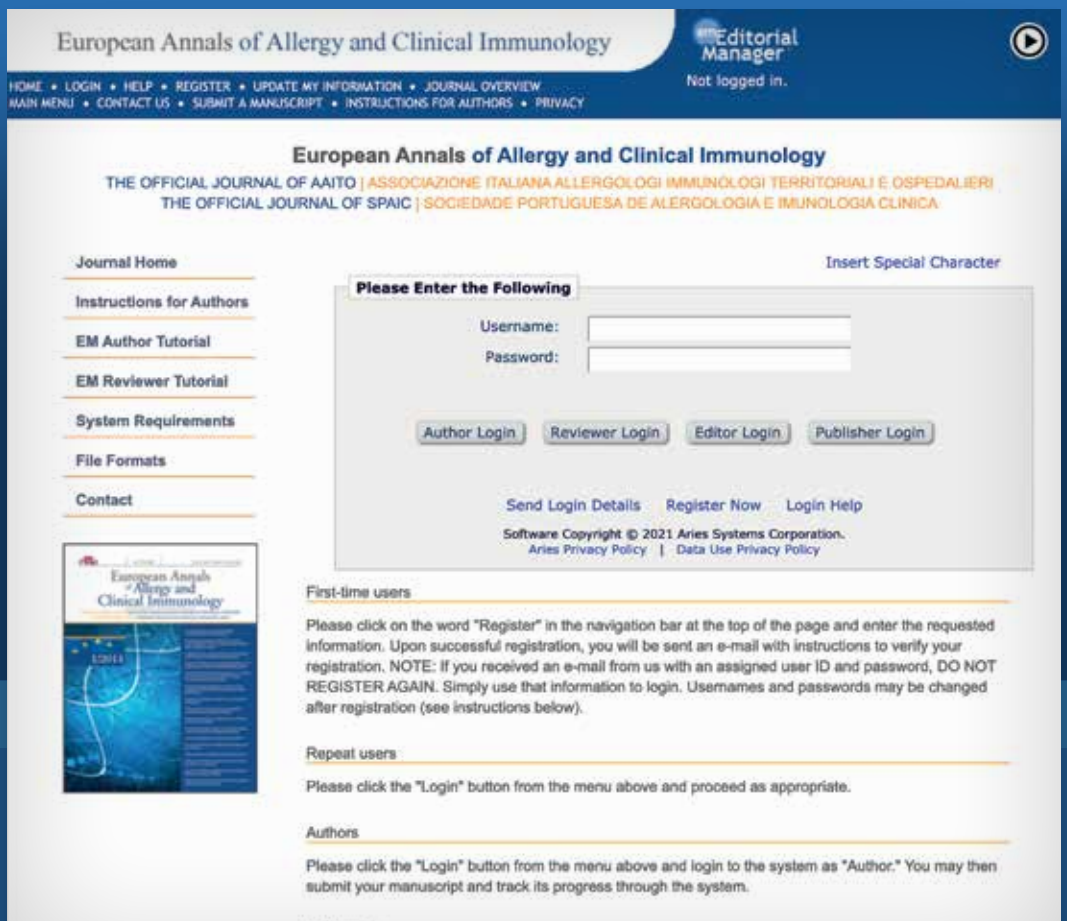

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Why lipid transfer protein allergy is not a pollen-food syndrome: novel data and literature review

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Key words

Food allergy; lipid transfer protein; pollen allergy; cross-reactivity; peach allergy.

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Summary

Background. *Based on the cross-reactivity between pollen lipid transfer proteins (LTPs) and the peach LTP, Pru p 3, it has been suggested that the pollen might initiate the LTP sensitization process.* ***Objective.*** *To establish whether LTP allergy can be considered as a pollen-food syndrome.* ***Methods.*** *The literature was reviewed and new data of component-resolved diagnosis from Italy obtained by both ISAC immunoassay and ImmunoCAP on large populations of LTP hypersensitive patients were provided and analyzed.* ***Results.*** *Among Pru p 3 reactors, patients positive for Art v 3 and Pla a 3 largely exceeded those sensitized to the respective major pollen allergens, Art v 1 and Pla a 1/Pla a 2. Pru p 3 reactivity remained stable around 80-90% at all ages, whereas Art v 3 and Ole e 7 recognition was missing in younger patients. Pru p 3 IgE exceeded IgE specific for pollen LTP at all ages. Inhibition studies carried out on LTP reactors showed that commercial extracts of mugwort and plane pollen were unable to inhibit significantly Pru p 3 IgE reactivity. In follow-up studies, baseline Pru p 3 IgE levels exceeded Art v 3 IgE levels in 84% of those sensitized to both allergens, and all patients positive to only one LTP allergen at baseline were sensitized to Pru p 3. Further, most of the patients who did not show any LTP reactivity at baseline became exclusive Pru p 3 reactors. On ImmunoCAP singleplex Pru p 3 IgE levels exceeded Art v 3 IgE levels in 89% of cases ($p < 0.0001$). Most literature data were in keeping with these new observations.* ***Conclusions.*** *The evidence for LTP syndrome being a pollen-food syndrome is presently very thin. Our data do not rule out the possible sensitization to the protein, via the airways or the skin.*

Impact statement

Both published data and new data rule out that LTP allergy derives from a pollen allergy

Introduction

A pollen food syndrome is the occurrence of a food allergy following primary sensitization to a phylogenetically conserved pollen allergen that is homologous (and hence, cross-reacting) to the relevant food allergen protein. There are several examples of pollen food syndrome in allergy. The best known is the cross-reactivity between the major birch pollen allergen Bet v 1, a PR-10, and homologous allergens in several fruits and vegetables (1). Other examples include sensitization to the pollen pan-allergen profilin which generally starts from grass pollen but can be triggered also by other sources such as birch or ragweed (2), the so-called mugwort-celery-spice syndrome, characterized by the primary sensitization to a minor mugwort allergen (3), and the food allergy to gibberellin-regulated proteins, that follows sensitization to a minor cypress pollen allergen (4). Two main in-vitro criteria must be fulfilled to define which the primary sensitizer among cross-reactive allergens is: 1) IgE level to the primary sensitizer is higher than IgE to the cross-reacting allergens; and 2) Cross-inhibition experiments show complete inhibition of the cross-reactive allergen when the primary sensitizer is used as an inhibitor, whereas the opposite does not happen (1-4). This because in most cases the primary sensitizer shows a larger number of IgE reactive epitopes than the cross-reacting allergen.

Non-specific Lipid Transfer Protein (LTP) is the most frequent cause of systemic allergic reactions induced by foods in the Mediterranean area (5). Its phylogenetically conserved nature and widespread distribution in the plant kingdom potentially expose hypersensitive patients to react to several botanically unrelated plant-derived foods. The peculiar geographical distribution of this type of food allergy, which is frequent in Southern Europe, rare north of the Alps, and virtually never described outside Europe except in China (6), has prompted the search for a putative airborne "primary sensitizer" ever since. Over the years, the major candidates for the role of "primary sensitizer" to LTP have been planetree, mugwort, and olive tree pollen due to their content in the three LTPs, Pla a 3, Art v 3, and Ole e 7, respectively. The presence of some degree of cross-reactivity between these pollen LTPs and Pru p 3, the peach LTP that is generally considered as the starting point for LTP sensitization, have fueled an ongoing discussion about the possible role of these pollens in the sensitization process. The present article reviews the currently available literature regarding each single putative sensitizing pollens, and adds new data, concluding that the evidence for a pollen-derived sensitization to LTP is presently very thin.

Critical review of the literature

Mugwort (Artemisia vulgaris)

The first *in vitro* studies about the cross-reactivity between Art v 3 and Pru p 3 and about the hypothetical role of the former in the LTP sensitization process appeared 20 years ago. Those studies concluded that mugwort LTP shares some epitopes with the homologous peach allergen but lacks other "main ones". The inhibition assays showed an almost full inhibition of IgE binding when peach was used as an inhibitor, whereas mugwort pollen was able to inhibit only partially the IgE binding by the fruit LTP (7). Subsequently, the same authors (8), as well as others, based on other cross-inhibition experiments (9), confirmed this finding. In the study by Pastorello and co-workers (9) the absorption of sera with as few as 4 μg of Pru p 3 was sufficient to abolish IgE reactivity to Pru p 3 in a peach extract, while 40 μg of Art v 3 caused only a partial inhibition. Further, importantly no pollen (including grass, ragweed, pellitory and olive tree) at the concentrations of 0.4 and 0.04 mg were able to inhibit the IgE reactivity to Pru p 3 suggesting that Pru p 3 is the LTP showing the highest number of epitopes (**table I**).

Surprisingly enough, after two years the authors of the first two papers changed their mind stating, based on new *in vitro* inhibition experiments, that Art v 3 behaves as a primary sensitizer in some patients with IgE to both Pru p 3 and Art v 3 (10). Some years later, an *in vivo* and *in vitro* Italian study tackled this view reporting that in Pru p 3 hypersensitive subjects skin tests with Artemisia pollen extract scored positive only in a minority of cases and that in patients co-recognizing peach and mugwort LTPs the former showed always much more intense skin reactions and elevated IgE levels than the latter (11). Later on, the possibility of primary sensitization to LTP via mugwort pollen returned into the discussion as some Chinese studies showed that in that country mugwort pollen plays a dominant role as a primary sensitizer to LTP (6, 12). Further, one Spanish study (13) showed that Artemisia LTP (Art v 3) can elicit allergic respiratory symptoms, but also stated that sensitization occurs through cross-reactivity starting from the peach. Finally, one study from Italy (14) showed that only one-fourth of 286 Art v 3 reactors recognized Art v 1, the mugwort major allergen, thus suggesting against a primary pollen sensitization. Again, *in vitro* inhibition experiments showed only a partial inhibition (just more than 50%) by Art v 3 over Pru p 3 IgE reactivity (14).

One consideration of pollen distribution is also worth doing. It is generally accepted that Artemisia pollen is present all over Europe (15, 16), but less distributed if not virtually absent in southern areas of the continent (https://www.polleninfo.org/FI/en/current-data/pollen-load-map-of-europe.html). Thus, firstly, it seems rather odd that mugwort pollen (specifically Art v 3) may induce a primary sensitization to LTP only in the southern part of the continent. Secondly, it seems unlikely that exposure to mugwort pollen and prevalence of LTP allergy show an opposite gradient of distribution over Europe. Further, the (limited) cases of LTP hypersensitivity in northern Europe have been associated with conditions other than mugwort pollen sensitization, such as Cannabis use (17, 18). In the UK as well as in Central Europe, Pru p 3 remains the key allergen in LTP hypersensitive patients (19, 20). Therefore, the conclusion drawn in 2012 by Spanish

Table I *- Amino acid sequence identity (%), identical positions and similar positions of LTP from different pollen sources vs Pru p 3 (IUIS data).*

POLLEN	nsLTP	IDENTITY	IDENTICAL POSITIONS	SIMILAR POSITIONS
Platanus orientalis	Pla or 3	46.6%	55	20
Platanus acerifolia	Pla a 3	45.7%	54	21
Artemisia vulgaris	Art v 3	40.5%	47	25
Ambrosia artemisifolia	Amb a 6	26.7%	32	25
Parietaria judaica	Par j 2	18.8%	25	35
Parietaria judaica	Par j 1	14.8%	26	29
Olea europea	Ole e 7	4.3%	4	7

```
P81402 NLTP1_PRUPE      1  ---------------------------------ITCGQVSSALAPCIPYVRGGG-AVPPA  26
A9YUH6 A9YUH6_PLAOI     1  MAFSRVAKLACLLLACMVAT------APHAEAAITCGTVVTRLTPCLTYLRSGG-AVAPA  53
P0C088 NLTP_ARTVU       1  ---------------------------------ALTCSDVSNKISPCLSYLKQGG-EVPAD  27
O04004 NLTP6_AMBAR      1  MDCIRILWSVAVGLLLVSWR------PTMFAASPTCDTVQNILAPCAGFLTGQ--EPSKA  52
P55958 NLT21_PARJU      1  MRTVSMAALV-VIAAALAWTSSAEPAPAPAPGEEACGKVVQDIMPCLHFVKGEEKEPSKE  59
P43217 NLT11_PARJU      1  ---------------------------------QETCGTMVRALMPCLPFVQGKEKEPSKG  28
P81430 ALL7_OLEEU       1  ---------------------------------APSQSTVTALLTSCVSYIDDQ-------  21
                                                              : . :   :  *  ::

P81402 NLTP1_PRUPE     27  CCNGIRNVNNLARTTPDRQAACNCLKQLSASVPGVNPNNAAALPGKCGVH-IPYKI-SAS  84
A9YUH6 A9YUH6_PLAOI    54  CCNGVKALNNDAKTTPDRQAACGCLKTASTSISGIQLGNAASLAGKCGVN-LPYKI-SPT 111
P0C088 NLTP_ARTVU      28  CCAGVKGLND-------------------------------------------------  37
O04004 NLTP6_AMBAR     53  CCTGVNNLNNSRKTKADRVAVCNCIKELTKSIA-YDPKRMPLLSTKCGVK-PDFPAVDKN 110
P55958 NLT21_PARJU     60  CCSGTKKLSEEVKTTEQKREACKCIVRATKGISGIKNELVAEVPKKCDIK-TTLPPITAD 118
P43217 NLT11_PARJU     29  CCSGAKRLDGETKTGPQRVHACECIQTAMKTYSDIDGKLVSEVPKHCGIVDSKLPPIDVN  88
P81430 ALL7_OLEEU      22  ---------------------------------------------------------  21

P81402 NLTP1_PRUPE     85  TNCATVK-----------------------------------------  91
A9YUH6 A9YUH6_PLAOI   112  IDCSKVK-----------------------------------------  118
P0C088 NLTP_ARTVU      38  -----------------------------------------------  37
O04004 NLTP6_AMBAR    111  LDCSKLPV----------------------------------------  118
P55958 NLT21_PARJU    119  FDCSKIQSTIFRGYY---------------------------------  133
P43217 NLT11_PARJU     89  MDCKTVGVVPRQPQLPVSLRHGPVTGPSDPAHKARLERPQIRVPPPAPEKA  139
P81430 ALL7_OLEEU      22  -----------------------------------------------  21
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authors that "mugwort sensitization results from cross-reactivity with other LTP sensitizations, rather than being a primary sensitization or a co-sensitization" (21) seems the most reasonable one.

Olive tree (Olea europaea)

The olive tree pollen lipid transfer protein, Ole e 7, displays a sequence identity with plant food LTPs that has been found to range between 50% (22) and 20% (23). The geographical distribution of olive tree pollen in Europe, which is quite overlapping with that of LTP-induced food allergy, prompted to consider this plant as a possible primary source of LTP sensitization. Although the association between severe food allergy and sensitization to Ole e 7 has been described (24), two Spanish studies were unable to detect any correlation between peach and olive tree pollen in LTP hypersensitive subjects (25) and between food allergy and Ole e 7 (18), respectively. Nonetheless, recently the possibility of olive tree pollen being the primary sensitizer to LTP in regions with high exposure was put forward once more from Spanish authors based on *in vitro* inhibition assays (26). Although about 80% of Ole e 7 reactors score positive to at least one plant food LTP (27), the fact remains that most Pru p 3 hypersensitive patients do not show any IgE reactivity to olive tree pollen on *in vivo* testing (11).

Planetree (Platanus acerifolia)

Planetree pollen sensitization is frequent in Spanish food-allergic individuals (28), and the planetree pollen LTP, Pla a 3 cross-reacts to other pollen and food LTPs (21, 29). Although the cross-reactivity between Pla a 3 and Pru p 3 seems bi-directional (30), specific IgE levels to Pru p 3 are generally higher than those to Pla a 3 (30). Further, also in this case, only a fraction of Pru p 3 hypersensitive patients show plane tree pollen hypersensitivity in the clinical setting (11). Finally, one Spanish study found a high prevalence of profilin sensitization in patients with plane tree pollen sensitization and food allergy (31). In the case of the plane tree, maps of pollen distribution (https://www.polleninfo.org/FI/en/current-data/pollen-load-map-of-europe.html) are consistent with the putative distribution of LTP allergy in Europe. Even though plane tree pollen is polluting virtually all European

countries, including the London area where the largest case series of LTP allergy north of the Alps has been published (20), this is again not completely in favour of the "pollen food" hypothesis for LTP allergy. LTP allergy prevalence is higher in the Mediterranean countries than in continental Europe where exposure to plane tree pollen is as high, if not higher, as in the southern areas.

Cypress (Cupressus arizonica)

Based on its geographic distribution, cypress pollen is another putative candidate as a primary sensitizer to lipid transfer protein (32). Nowadays we know that cypress pollen is the primary sensitizer to gibberellin-regulated protein, which is associated with systemic reactions to different fruits, particularly the peach (33, 34). To our knowledge, there are no data regarding an association with food LTP hypersensitivity and besides, no LTPs have been identified in cypress pollen so far (http://www.allergen.org).

Pellitory (Parietaria judaica)

Despite pellitory is one of the major sources of aeroallergens in the Mediterranean areas (16) and therefore a putative sensitizer in the LTP allergy, this is not the case from both a clinical and molecular point of view. In a study on Mor m 3, the mulberry nsLTP (35), the Authors investigated the alignment of the amino acid sequences from Mor m 3 and other nsLTP (including Pru p 3, Art v 3, and Par j 2) evaluating the relevant regions showing IgE-binding activity in Pru p 3 *vs* other nsLTPs. Little amino acid identity was found in the sequence of the IgE-binding regions between Pru p 3 and both Art v 3 and Par j 2, suggesting that the two pollens cannot be considered responsible for the sensitization to Pru p 3.

Natural history

Another way to establish whether fruits (peach) or pollen is the "primary sensitizer" to LTP is to look both at the natural history and the epidemiological data of allergic diseases in patients included in the studies dealing with LTP allergy. Unfortunately, these aspects are not addressed in most cases. In an international study (36), apple allergy started later than pollen allergy in all 4 participating countries (Austria, Italy, Netherlands, and Spain), but while in the former three apple allergy followed the primary sensitization to birch pollen, in Spain apple allergy followed Pru p 3 hypersensitivity which in turn occurred at the same time as pollen allergy, with grass being by far the main one. Two further studies from Spain (25, 37) did not find any relationship between the prevalence of sensitization to Pru p 3 and any pollinosis.

Methods

Component resolved diagnosis in italian patients

Five allergy units (Milan, Palermo, Pavia, Pordenone, and Rome) scattered throughout the Italian territory provided their *in vitro* data obtained in 9138 allergic patients measuring IgE either by ImmunoCAP ISAC 112 or by singleplex ImmunoCAP (both Thermo Fisher Scientific, Uppsala, Sweden), between September 2015 and December 2020. All tests were performed during routine care, and the samples were anonymized, since no personal data, except for age and sex, was available. The Institutional Review Board of IDI-IRCCS confirmed that ethical approval was not required in this case (n. 493.1).

Serum IgE reactivity was analyzed using the latest commercially available ImmunoCAP-ISAC platform as per the manufacturer's instructions. In brief, ImmunoCAP-ISAC 112 slides were washed, rinsed and dried at room temperature (RT). Undiluted serum (30 µl) from each patient was pipetted on to the slide and after 120 min incubation at RT in a humid chamber, slides were washed, rinsed and dried. IgE binding was detected by the addition of an anti-human secondary antibody (ThermoFisher Scientific). Slides were then washed, rinsed, dried, and stored in the dark until scanning. Images were acquired by scanning allergen biochips with a CapitalBioLuxScan™ 10K microarray scanner. IgE values are expressed as ISU arbitrary units (ISAC Standardized Units) corresponding to IgE antibody levels in the ng/ml range (detection limit: 0.01 ISU-E, values above 0.3 ISU-E were considered as positive) (38). For the follow-up studies, since in some cases the comparisons were made with versions of the ISAC test containing a lower number of LTPs, the serial evaluations were performed only for Art v 3 and Pru p 3.

Sera from Palermo were tested with the singleplex ImmunoCAP 250 following the manufacturer's instructions and the selected cut-off value was 0.1 kU/L.

Statistics

All data were analyzed with the IBM SPSS statistical package version 21 (Armonk, NY). The TD-Synergy Laboratory Information System was used to search and collect demographic information, *i.e.*, age and gender, and clinical and laboratory data for patients who attended the outpatient Allergy clinic and underwent specific IgE testing. Categorical variables were analyzed using Pearson's χ^2 or Fisher's exact test. Differences between prevalences were evaluated using the nonparametric Mann–Whitney U-test. The degree of relationship between quantitative variables was analyzed using Spearman's correlation (r) coefficient, given the non-parametric distribution of the observed values. Separate modelling was performed for each condition including all molecules, in addition to sex and age. P-values < 0.05 were considered significant.

Results

ISAC Immunoassay data

Prevalences and IgE levels

IgE levels to Pru p 3, Art v 3, Ole e 7, and Pla a 3 were measured in 2048 LTP-hypersensitive patients (age 30 ± 16, 1136 F). Among

Pru p 3 reactors, the number of patients positive for Art v 3 and Pla a 3 largely exceeded that of patients sensitized to the respective major pollen allergens, Art v 1 and Pla a 1/Pla a 2, which are generally considered as markers of genuine pollen sensitization (**table II**), suggesting that both Art v 3 and Pla a 3 sensitizations were the result of a cross-reactivity in which Pru p 3 acts as the primary sensitizer.

Table II *- The proportion of patients positive for Art v 1, Art v 3, Ole e 1, Ole e 7, and Pla a 1-3 among patients not showing or showing IgE reactivity to Pru p 3.*

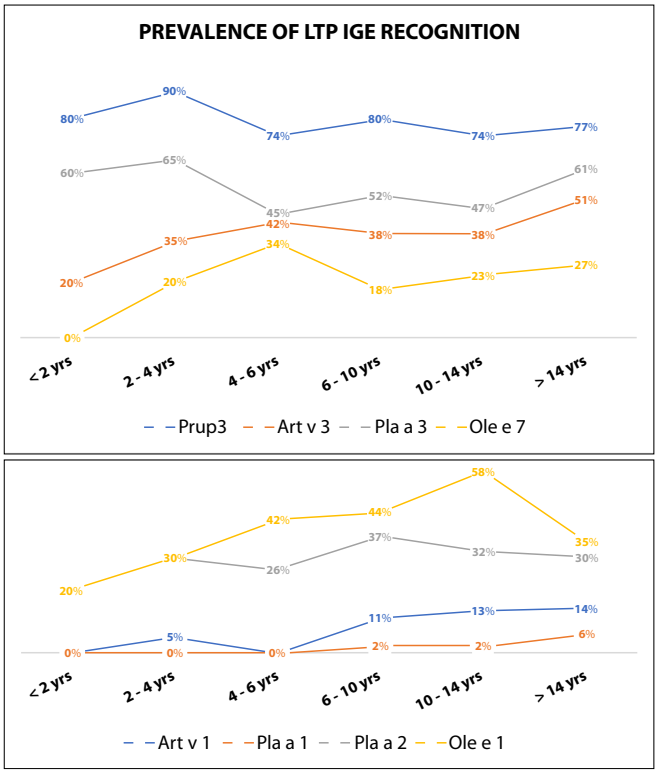
	Pru p 3^{neg} (466)	**Pru p 3^{pos} (1582)**
	% within the respective subset	
Art v 1	21.9%	11.1% *
Art v 3	21.2%	57.0%*
Ole e 1	44.6%	34.6%*
Ole e 7	31.1%	24.5%*
Pla a 1	4.3%	5.4%
Pla a 2	28.3%	31.0%
Pla a 3	26.2%	69.7%*

*< 0.01. The comparisons were carried out by the z test. Tests are adjusted for all pairwise comparisons within a row of each innermost subtable using Bonferroni's correction.

Further, the age distribution of pollen nsLTP molecules throughout the entire population showed that the prevalence of Pru p 3 recognition remained stable around 80-90%, whereas Art v 3 and Ole e 7 recognition were missing in patients younger than two years of age, and progressively increased in older children to reach the adult level after 6 years. On the other hand, Pla a 3 was regularly recognized in about one half of the population in all age subsets observed. Overall, Pru p 3 IgE recognition exceeded, if not doubled, the IgE recognition of the pollen LTP molecules in all the age subsets considered, making it very difficult to hypothesize that the latter could act as sensitizing molecules in the Mediterranean population studied (**figure 1**).

The mean levels of IgE to a series of different LTPs including also Ara h 9, Cor a 8, Jug r 3, and Tri a 14 were calculated and plotted against the presence or absence of Pru p 3 IgE reactivity. The mean specific IgE levels increased significantly in the presence of Pru p 3 reactivity in all cases except for Tri a 14 and Ole e 7, which did not change (**table III**). The linear correlation between Pru p 3 IgE levels and IgE levels of all other LTPs studies was significant at 0.001 (2-tailed) in all cases (Spearman's rank correlation coefficient between Pru p 3 and Ara h 9: 0.781; Art v 3: 0.720; Cor a 8: 0.735; Jug r 3: 0.830; Ole e 7: 0.399; Pla a 3: 0.798).

Figure 1 *- (**A**) Prevalence of IgE recognition of several LTPs in pediatric patients at different ages. (**B**) Major pollen allergens Ole e 1, Pla a 1, Pla a 2, and Art v 1 trend of IgE prevalence in the same population.*



Inhibition studies

IgE reactivity to Art v 3, Pla a 3, and Pru p 3 of sera from 3 patients sensitized to all three allergens were measured before and after absorption of sera with commercial extracts of Artemisia vulgaris and Platanus acerifolia (Stallergenes, Anthony, France). Inhibition < 75% of IgE reactivity was arbitrarily considered as not relevant. Results are shown in **figure 2**. In no case, the two commercial extracts were able to induce significant inhibition of Pru p 3 IgE reactivity, whereas this was often the case for IgE reactivity to Pla a 3 and Art v 3.

Follow-up data

IgE to Pru p 3 and Art v 3 were measured serially in 102 pediatric (age range 6 mo-6 years) patients. Measurements were carried out at intervals of at least one year; 85, 11 and 6 patients had 2, 3 and 4 measurements, respectively. Based on baseline findings these patients were divided into 3 subgroups:

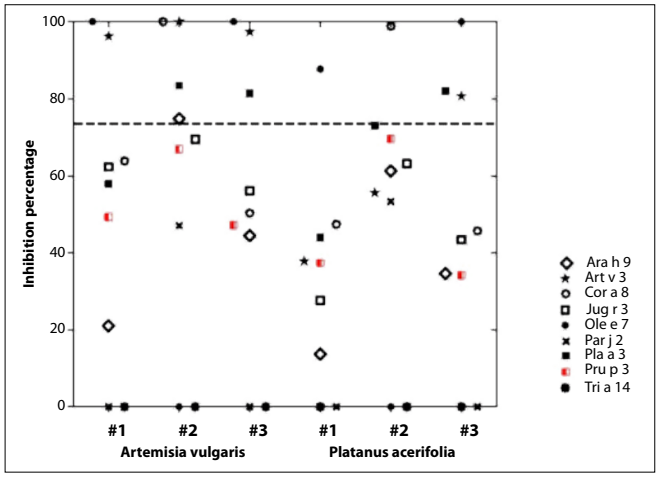
- Patients who showed IgE to both LTPs at baseline (n = 19).
- Patients who showed IgE to one of the two allergens (n = 60).
- Patients who did not show IgE to any of the two allergens (n = 23).

Table III *- Comparison between the mean IgE levels to several LTPs in the presence or the absence of Pru p 3 sensitization.*

	Pru p 3^{neg} - lgE Mean ± Standard Deviation	**Pru p 3^{pos} (3.79 ± 7.59 ISU)**
Ara h 9	0.14 ± 0.61	1.55 ± 3.21*
Art v 3	0.33 ± 2.08	1.4 ± 3.38*
Cor a 8	0.06 ± 0.32	1.11 ± 2.98*
Jug r 3	0.27 ± 1.11	2.28 ± 4.1*
Ole e 7	1.87 ± 9.08	0.99 ± 5.98
Pla a 3	0.42 ± 1.74	1.72 ± 3.59*
Tria14	0.15 ± 1.47	0.45 ± 2.47

*< 0.01. The comparisons were carried out by the z test. Tests are adjusted for all pairwise comparisons within a row of each innermost subtable using Bonferroni's correction.

Figure 2 *- Inhibition of IgE reactivity to Pru p 3, Art v 3 and Pla a 3 by commercial mugwort and plane tree extracts.*



Subgroup a: in patients reactive to both LTPs, baseline Pru p 3 IgE levels exceeded Art v 3 IgE levels in 16/19 cases (84%) (median levels 3.09 *vs* 1.4 ISU-E, respectively). At the follow-up observations, Pru p 3 IgE levels exceeded Art v 3 IgE levels in 15/19 cases (78%), including 2/3 of those showing higher Art v 3 levels at baseline.

Subgroup b: all patients positive for only one of the two LTPs at baseline scored positive for Pru p 3 (100%). At the follow-up analyses, 27 (45%) were still monosensitized to Pru p 3 while 33 (55%) had become positive to Art v 3 also, although IgE levels to Pru p 3 exceeded Art v 3 IgE levels in 30/33 (90.9%) cases.
Subgroup c: of 23 patients who did not show any LTP reactivity at baseline and had become LTP reactors at the first follow-up control, 13 (56.5%) were exclusive Pru p 3 reactors, 9 (39.1%) reacted to both Pru p 3 and Art v 3 (with Pru p 3 IgE exceeding Art v 3 IgE in 8 cases, while in 1 case the levels were identical), whereas the remaining patient showed elevated levels of Art v 3 IgE but no reactivity to Pru p 3.

ImmunoCAP data

Data from 285 consecutive LTP-sensitized patients (mean age 38.2 years; range 2-79; 184 F) collected in Palermo were evaluated using the singleplex ImmunoCAP. Of these, 275 (96.5%) were Pru p 3 reactors, and 200 (70%) showed IgE to Art v 3. IgE reactivity to other food LTP including Ara h 9 (80.7%), Jug r 3 (82.5%), Tri a 14 (57.2%) and Cor a 8 (68.8%) are summarized in **table IV**. Data from further 3.026 patients (mean age 34.1 years; range 3-74;1104 males, 1922 females), tested for Pru p3, *Parietaria judaica* and *Olea europea* extracts were also analyzed. No significant relationship between the allergens tested was found (Concordance correlation coefficient Pru p 3 - *Olea europea* = 0.348; Pru p 3 - *Parietaria judaica* = 0.322).

Discussion

The concept of pollen-food allergy syndrome implies the primary sensitization to a seasonal aeroallergen which is followed by a food allergy caused by the homology between one or more pollen allergens with one or more food proteins. Apple or hazelnut allergy in birch pollen allergic patients represent a perfect example in this sense, and nobody could reasonably claim that apple is the primary sensitizer despite apple IgE can be detected in the majority of birch pollen-allergic patients (1, 39).
In the case of allergy to LTP, things appear completely different. Available data, including the new *in vitro* data that we reported here, seem to rule out the sensitization to a pollen source as the starting point of LTP syndrome unless one postulates that peach LTP allergy is the result of the sensitization to any pollen LTP among planetree, mugwort, olive tree, or pellitory all leading to the same eventual food allergy. Furthermore, the lack of cross-reactivity between Ole e 7 and/or Par j 1-2 sensitization and Pru p 3 has already been described in the literature (23), mainly due to the widely known structural difference between such LTPs. Inhibition as well as prevalence data seem to rule out this possibility. Inhibition studies have been performed with only 3 sera, but the inability of planetree or mugwort extracts to completely inhibit the Pru p 3 signal in all cases can be con-

Table IV *- Serological data of 285 LTP sensitized subjects.*

	IgE level	n. positive patients (%)	χ^2	Significance
Pru p 3 (6.06 ± 11.86)	> Art v 3 (2.25 ± 6.62)	253 (89.4%)	357.202	p < 0.0001
	> Jug r 3 (4.36 ± 12.80	244 (86.22%)	301.098	
	> Tri a 14 (1.56 ± 4.38)	256 (90.46%)	383.046	
	> Ara h 9 (4.08 ± 10.36	242 (85.51%)	308.388	
	> Cor a 8 (2.00 ± 5.63)	251 (88.69%)	357.255	

The table shows the values (and percentages) of those patients who had specific IgE levels towards the nsLTPs evaluated by ImmunoCAP with values lower than those found for Pru p 3.

sidered as indirect evidence that neither planetree nor mugwort act as the primary sensitizers in patients with LTP allergy. In all cases studied, pollen LTP allergens seem to show less allergenic epitopes than peach LTP, and IgE levels are in favour of peach LTP in most cases.

The peculiar geographic distribution of LTP allergy points to a local (Mediterranean) trigger. Of course, we cannot exclude *tout court* the primary airborne sensitization to a hitherto unknown pollen source although this hypothesis seems unlikely if one considers that a large proportion of LTP allergic patients score completely negative on allergic testing for all seasonal airborne allergens and do not report any respiratory allergy. However, several data have accumulated over the years suggesting a possible direct sensitization to peach LTP via the airways (40-43) or the skin (44-46). Again, this does not explain the geographic prevalence of this allergy, although one has to consider that for instance peach fuzz is removed from the fruits to be exported in countries where peaches are not grown (40). The main producers of peaches in the world are China, Italy, Greece, Spain, and the USA (47). Interestingly, except for the USA, these countries represent the areas showing the highest prevalence of LTP allergy.

Conclusions

In conclusion, we believe that the data available to date, including those of the present study, point against a primary pollen sensitization in LTP allergic patients.

Fundings

None.

Conflict of interests

The authors declare that they have no conflict of interests.

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Severe cutaneous adverse drug reactions: diagnostic approach and genetic study in a Brazilian case series

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Key words

Drug allergy; genetics; immunologic tests; pharmacology; pharmacogenomics.

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Impact statement

The study is the first case series of SCAR with HLA analysis from Brazil. It had shown the occurence of the various described alleles of risk all over the world among our mixed population. The finding of allopurinol, carbamazepine and abacavir related HLA alleles combined with patch test positivity to anticonvulsants reinforced the culprit drug.

Summary

Background. *Severe cutaneous adverse reactions (SCAR) are potentially fatal reactions. Genetic predisposition is involved in their pathogenesis related to drugs and ethnicities, however in a mixed population these relationships are still unknown. The aim of this study was to describe phenotypes, suspect drugs and HLA-alleles related to SCAR, identified by a systematized approach in a Brazilian case series.* ***Methods****. Patients who were diagnosed with SCAR between March 2011 and July 2019 at our university hospital were included. European Network for Drug Allergy (ENDA) questionnaire was used to collect clinical and laboratory data and algorithms for assessment of drug causality were applied. Socio-demographic variables included age, gender and skin color/ethnicity. Drug patch tests (DPT) and HLA-A, -B, -DRB1 typing were carried out.* ***Results****. A total of 74 patients were included: 36 (48.64%) with SJS/TEN, 32 (43.24%) DRESS/DIHS, 3 (4.05%) AGEP, 2 (2.70%) overlap (DRESS/SJS and DRESS/AGEP) and 1 (1.35%) GBFDE. The median age was 31.5 years (IQR = 14-52.25), most were female (n = 44/59.46%) and brown (n = 38/51.35%). Anticonvulsants (n = 32/43.24%) were the largest group involved and antibiotics (n = 26/35.13%) were the second most common. Two patients with DRESS died during the acute phase. Positive DPT were shown only in anticonvulsant associated DRESS. HLA related to abacavir, allopurinol and carbamazepine were identified.* ***Conclusions.*** *A systematized approach allowed the phenotypic characterization of SCAR. The HLA-A*31:01, B*57:01 and B*58:01 alleles were identified, reinforcing the causality in SCAR by CBZ, ABC and ALLO in the Brazilian population.*

Introduction

Severe cutaneous adverse reactions (SCAR) are rare, delayed type, life-threatening hypersensivity drug reactions that include four phenotypes: Stevens Johnson syndrome/toxic epidermal necrolysis (SJS/TEN), drug reaction with eosinophilia and systemic symptoms/drug induced hypersensitivity syndrome (DRESS/DIHS), acute generalized exanthematous pustulosis (AGEP) and more recently, generalized bullous fixed drug eruption (GBFDE) (1). Incidence of SCAR reaches 2% in hospitalized patients, with mortality rate between 5 and 50% (2). The development of sequelae with variable degrees of morbidity and incapacity has a direct impact on quality of life (3). Their high morbidity and mortality highlight the importance of rapid diagnosis and immediate withdrawal of the suspect drug (4, 5). Clinical diagnosis obeys the multinational registry of SCAR (RegiSCAR) and grading system criteria, while the etiology is presumed by chronological criteria, drug notoriety and application of causality algorithms (1). The drug patch tests (DPT) are safe and may be useful to ratify the etiology when positive and intradermal skin tests (IDT) can be done in selected cases. Oral provocation test (OPT) is contraindicated (6).

In clinical practice, HLA typing is a useful tool for screening genetically susceptible individuals for only a few drugs for which cost-effectiveness studies have already been delineated (7,8). It can also be used for differential diagnosis of bedside SCAR in patients using highly related medications. However, the relevance of such alleles in the Brazilian mixed population is unknown.

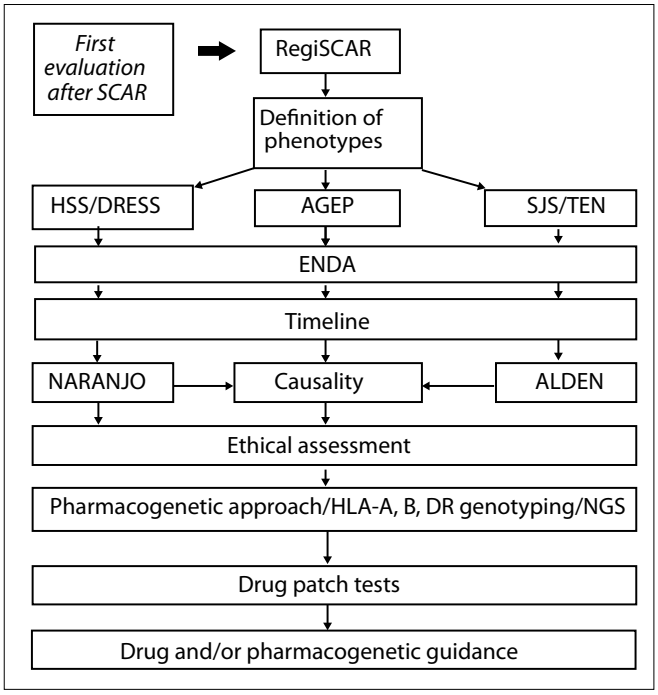
Methods

Cross-sectional, retrospective study, based on medical records of SCAR patients referred to the Adverse Drug Reaction (ADR) Ambulatory of Rio de Janeiro State University/Brazil (UERJ), between March 2011 and July 2019. In order to identify DRESS/DIHS, AGEP, SJS/TEN and GBFDE we used the RegiSCAR criteria (1). In patients diagnosed in other hospitals, a copy of the hospitalization record was requested to analyze clinical data. Demographic variables analyzed were gender, age and skin color/ethnicity, obtained by the self-attribution method (9).

Clinical approach in all cases included the application of ENDA questionnaire, adapted to Brazilian portuguese (10, 11) and a timeline to register clinical and laboratorial data. Patients suspected of DRESS/DIHS were submitted to the DRESS probability classification (12). The European Study of Severe Cutaneous Adverse Reactions (EuroSCAR) scoring system established a score for possible, probable and defined cases of AGEP (13). In SJS/NET, the degree of epidermal detachment was used to classify cases in three groups: 1-10% as SJS, 10-30% overlap SJS/TEN and > 30% as TEN (14). GBFDE was considered when well-demarcated dusky red or heavily pigmented patches with blisters and erosions involving the skin and mucosa were seen (1). Overlap forms of SCAR were suspect if the criteria for definite or probable diagnosis of, at least, 2 SCARs were filled (15). The timeline and causality algorithms were used to define culprit drugs. The clinical judgment of the attending physician was proposed based on the latency period, drug notoriety and characteristics of each phenotype (16). For all reactions the Naranjo Scale (17) was applied. For Stevens-Johnson syndrome/toxic epidermal necrolysis, the algorithm for assessment of drug causality in Stevens-Johnson syndrome/toxic epidermal necrolysis (ALDEN) was used (18). When the patient was consuming more than one suspected drug, it was considered the one that presented the highest score in algorithms and if the score was similar, both drugs were considered together.

In addition, we performed DPT, according to the European Society of Contact Dermatitis (ESCD) guidelines (19) and DNA–based typing (HLA-ABDR). A diagnostic algorithm was developed for the routine systematized approach of patients (**figure 1**). DPT were performed at least six months after complete healing of reaction and one month after discontinuation of systemic corticosteroid. Tests were conducted using diluted doses in white petrolatum up to 10% with the active ingredient and up to 30%

Figure 1 *- Diagnostic algorithm.*



with the commercialized form. Petrolatum was used as control. DPT were manufactured in authorized pharmacy. Finn Chambers on Scanpor tape (Smart Practice Phoenix, AZ, USA) were used and results were reported according to the International Contact Dermatitis Research Group (ICDRG) criteria on days 2 and 4. If the patch tests were negative on day 4, additional reading was carried out on day 7. Healthy volunteers without prior exposure to the suspected drug were tested as negative controls in all DPT and standard DPT were negative with the used vehicles. Patients were submitted to peripheral venous blood collection in our institution Clinical Pathology Service (Capsula). The material was used to HLA-A, -B, -DRB1 typing by PCR-RSSO (One Lambda, Canoga Park, CA/USA), which was performed in the majority of patients. In cases in which drug-related risk alleles were identified, AllTypeTM next generation sequencing (NGS 11_Loci Amplification Kit – One Lambda, Canoga Park, CA/USA) was performed. Pharmacogenetic advice was given for patients with well-defined risk alleles and their close relatives.

Descriptive analyzes were performed using Microsoft Office Excel 2010 (Microsoft Co., WA/USA). The dichotomous variables were described by numbers and percentages, and the continuous variables by median, interquartile range (IQR) and/or mean (average; minimum-maximum).

The study was approved by the local ethical committee. Signed informed consent of controls and patients were acquired.

Results

A total of 74 patients were included: 36 SJS/TEN (48.64%), 32 DRESS/DIHS (43.24%), 3 AGEP (4.05%), 2 (2.70%) overlap cases (DRESS/SJS and DRESS/AGEP) and 1 GBFDE (1.35%). The median age was 31.5 years (IQR = 14-52.25) and the majority were female (n = 44/59.46%). Regarding skin color, 38 (51.35%) were brown, 18 (24.32%) black and 16 (21.62%) white, and only two were Asian. All patients had a probable score on Naranjo scale. The median latency period was 15 days (IQR = 8-26.25) (**table I**). Aromatic anticonvulsants (ACA), including carbamazepine (CBZ), phenytoin (PHT), phenobarbital (PB) and lamotrigine (LMT) were involved in more than half of the reactions, followed by antibiotics with predominance of beta-lactams. Analgesics/NSAID, allopurinol, among others, were the remaining culprit drugs (**table II**).

Patients with SJS/TEN spectrum presented as SJS (n = 22/61.11%), overlap SJS/TEN (n = 8/22.22%) and TEN (n = 6/16.66%). DRESS/DIHS was the second most frequent SCAR. Kardaun's classification for DRESS scored as probable (n = 19/59.37%), definite (n = 12/37.5%) and possible case (n = 1/3.12%). All of them presented hepatic involvement and significant eosinophilia was the most common haematological finding. Four patients presented disease reactivation and were readmitted to the hospital after apparent control of the clinical picture. Three patients with AGEP were identified. All were female, had a probable EuroSCAR score for AGEP and good response to systemic corticosteroids. A 21-year-old male had GBFDE (1.35%). He presented 3 episodes of pharmacodermia after use of dipyrone, with reactivation of residual hyperpigmented lesions and appearance of new lesions on the face, trunk, oral mucosa and genitalia.

Overlap SCAR probability criteria were identified in a 50-year-old female with definitive diagnosis of allopurinol (ALLO) induced DRESS and probable AGEP according to RegiSCAR/EuroSCAR score. Another case of SCAR overlap was identified in a SJS/TEN 82 years old black woman using naproxen for 10 days. ALDEN scored very probable, but she also had fever, eosinophilia and liver involvement making DRESS a probable diagnosis by RegiSCAR.

Only 30 patients authorized the DPT: DRESS and DRESS/AGEP overlap (n = 14), AGEP (n = 2) and SJS/TEN (n = 14). Most of them were done with the commercialized form (n = 22/73.33%) and the remaining with pure substances provided by hospital pharmacy. Positive DPT were seen only in anticonvulsants-induced DRESS/DIHS: five with CBZ up to 10% dilution and 1 with 30% dilution of PB. The positivity of DPT for CBZ in DRESS/DIHS patients was 85.33% (**table III**). A CBZ-induced DRESS patient with negative DPT at 5% had rash and fever without organ involvement and the investigation was not continued. None of the other patients presented reactions related to tests.

The HLA-A, -B, -DRB1 typing was performed in 67 patients (90.54%). Risk alleles related to abacavir (ABC), ALLO and CBZ have been identified in eight (11.94%) out of sixty-seven patients who underwent genetic testing (**table IV**).

Discussion

In our series, as in other publications of SCAR, women were more affected (19, 20). Most of SCAR patients presented the SJS/TEN spectrum. The median age was lower than in other studies and reflects the characteristic of our Service, where we care adults and children. The predominance of brown skin color reflects the characteristic miscegenation of Brazilian population. Diagnosis of SCAR is eminently clinical and represents a challenge since it includes a variety of differential diagnoses. The use of criteria established by Euro/RegiSCAR is of paramount importance. The ENDA questionnaire and the timeline were valuable auxiliary tools in the SCAR patient approach (10). The visualization of the chronology of the clinical-laboratory data and use of the suspicious drugs allowed a better characterization of SCAR.

The skin rash biopsy may be helpful because it presents typical findings in the cases of SJS/TEN and AGEP, however it is non-specific in DRESS/DIHS (21). In this study, it was useful in an overlapping phenotype (DRESS/AGEP) in a patient with DRESS criteria. Although overlapping of different skin lesion patterns is not uncommon among phenotypes, true cases of SCAR overlap seem to be rare (15).

The high index of suspicion for drug involvement is crucial because the immediate suspension of suspected and non-essential drugs is a determining factor for prognosis (22, 23). A long latency period delays the suspension of the culprit drug and causality can be falsely attributed to drugs used to treat reaction prodromes, on the other hand, drugs used for long time should not be forgotten especially if used irregularly or intermittently (16, 24, 25). In this study, one patient with ALLO associated SJS/TEN and another PB related DRESS patient presented SCAR after one year of irregular drug use.

Causality algorithms were applied in the approach of all patients. Naranjo's criteria (17) cover general aspects of drug reactions, and, in this study, it often did not reach high or low imputability scores, while ALDEN (18), developed for epidermal necrolysis, allowed us to identify and/or exclude some medications as an etiological factor in most patients.

As in the majority of studies, anticonvulsants, antibiotics, NSAID and ALLO were the main classes of drugs involved (16, 20, 26, 27). The group of ACA was involved in most of our cases of DRESS/DIHS. Among ACA, CBZ followed by PHT was the major involved in DRESS. In SJS/NET group, anticonvulsants were also more involved in the etiology of the disease, but antimicrobials (especially beta-lactams) represented the second largest group. The significant higher risk associated with concomitant use of valproic acid and lamotrigine, due to increased half-life of lamotrigine elimination, was identified in an overlap SJS/NET case (28).

Table I *- Demographic, clinical, and laboratory characteristics of patients.*

Characteristic	SJS/TEN (n = 36)	DRESS (n = 32)	AGEP (n = 3)	GBFDE (n = 1)	Overlap (n = 2)
Age (years)				21	
median (IQR)	29.5(13.75-46)	31 (12.75-53.75)	-	-	-
average (min-max)	-	-	42 (16-55)	-	66 (50-82)
Skin color/ethnicity n (%)					
Brown	16 (44.44)	19 (59.37)	2 (66.66)	1 (100)	-
White	8 (22.22)	8 (28.12)	1 (33.34)	-	-
Black	11 (30.55)	3 (9.37)	-	-	2 (100)
Yellow	1 (2.77)	1 (3.12)	-	-	-
Sex n (%)					
Female	21 (58.33)	14 (43.75)	3 (100)	-	2 (100)
Male	15 (41.66)	18 (56.25)	-	1 (100)	-
Risk fator n (%)					
Autoimmunity	4 (11.11)	3 (9.37)	1 (33.33)	-	1 (50)
HIV serology	2 (5.55)	2 (6.25)	-	-	-
HLA risk allele presence	4 (11.11)	3 (9.37)	-	-	1 (50)
Latency period (days)	-			2	
median (IQR)	15 (6.75-22.5)	16.5 (15-28.5)		-	
average (min-max)	-		12.5 (2-15)		15 (10-20)
Clinical findings n (%)					
Skin involvement	36 (100)	32 (100)	3 (100)	1 (100)	2 (100)
Mucosal involvement	36 (100)	6 (18.75)	-	1(100)	1 (50)
Lymphadenopathy	3 (8.30)	19 (59.37)	1 (33.33)	-	1 (50)
Fever ≥ 38 ºC	33 (91.66)	31 (96.87)	2 (67.70)	1 (100)	2 (100)
Hematologic findings, n (%)					
Eosinophilia	-	25 (78.12)	-	-	2 (100)
Neutrophilia (> 7,000)	-	-	3 (100)	-	1 (50)
Involved organs n (%)					
Liver	6 (16.66)	31 (96.87)	-	-	2 (100)
Gastrointestinal	1 (2.77)	5 (15.62)	-	-	-
Kidney	6 (16.66)	6 (18.75)	-	-	-
Lung	5 (13.88)	8 (32.0)	1 (33.33)	-	1 (50)
Heart	5 (13.88)	6 (18.75)	-	-	-
SNC	2 (5.55)	3 (9.37)	-	-	-
Treatment n (%)					
Systemic corticosteroid	24 (66.66)	27 (84.37)	3 (100.0)	1 (100)	2 (100)
Systemic corticosteroid + IGIV	6 (16.66)	3 (9.37)	-	-	-
IGIV	4 (11.11)	-	-	-	-
Supportive care only	2 (5.55)	-	-	-	-

→

Characteristic	SJS/TEN (n = 36)	DRESS (n = 32)	AGEP (n = 3)	GBFDE (n = 1)	Overlap (n = 2)
Treatment time (days)			-	18	-
median (IQR)	24 (5-52.5)	120 (30 -180)	-	-	-
average (min-max)	-	-	36 (4-90)	-	55 (20-90)
Inpatient stay (days)	-	-	-	10	-
median (IQR)	19.5 (13-29.5)	15 (12-30)	-	-	-
average (min-max)	-		2.5 (1-4)		16.5 (12-21)
Death n (%)	-	2 (6.25)	-	-	-

SJS: Stevens-Johnson Syndrome; TEN: Toxic Epidermal Necrolysis; DRESS: Drug Reaction with Eosinophilia and Systemic Symptoms; AGEP: Acute Generalized Exanthematous Pustulosis; GBFDE: Generalized Bullous Fixed Drug Eruption; IQR: interquartile range; HLA: human leukocyte antigen; IGIV: intravenous immunoglobulin.

Table II *- Etiology of SCAR.*

Drugs	DRESS/DIHS n = 32 (%)	SJS/TEN n = 36 (%)	AGEP n = 3 (%)	GBFDE n = 1 (%)	Overlap n = 2 (%)	Total n = 74 (%)
Aromatic anticonvulsants	20 (62.5)	23 (63.88)	0 (0.00)	0 (0.00)	0 (0.00)	43 (58.11)
Carbamazepine	8	5	0	0	0	13 (17.57)
Lamotrigine	1	5	0	0	0	6 (8.10)
Oxcarbazepine	1	0	0	0	0	1 (1.35)
Phenobarbital	2	7	0	0	0	9 (12.16)
Phenytoin	8	6	0	0	0	14 (18.92)
Antibiotics	5 (15.62)	22 (61.11)	1 (33.33)	0 (0.00)	0 (0.00)	28 (37.83)
Azithromycin	0	1	0	0	0	1 (1.35)
Amoxicilin	0	3	1	0	0	4 (5.40)
Ampicilin	0	1	0	0	0	1 (1.35)
Benzathin penicilina	0	1	0	0	0	1 (1.35)
Meropenem	0	3	0	0	0	3 (4.05)
Cefaclor	0	1	0	0	0	1 (1.35)
Cefalexine	1	0	0	0	0	1 (1.35)
Cefepime	0	1	0	0	0	1 (1.35)
Ceftriaxone	1	1	0	0	0	2 (2.70)
Chloramphenicol	0	1	0	0	0	1 (1.35)
Gentamicina	0	1	0	0	0	1(1.35)
Quinolone	0	2	0	0	0	2 (2.70)
Sulfamethoxazole	2	3	0	0	0	5 (6.75)
Tetracycline	0	1	0	0	0	1 (1.35)
Vancomycin	1	2	0	0	0	3 (4.05)
Antiviral drugs	1 (3.12)	2 (5.55)	0 (0.00)	0 (0.00)	0 (0.0)	3 (4.05)
Abacavir	1	0	0	0	0	1 (1.35)
Nevirapine	0	2	0	0	0	2 (2.07)

Drugs	DRESS/DIHS n = 32 (%)	SJS/TEN n = 36 (%)	AGEP n = 3 (%)	GBFDE n = 1 (%)	Overlap n = 2 (%)	Total n = 74 (%)
Allopurinol	2 (6.25)	7 (19.44)	1 (33.33)	0 (0.00)	1(50.0)	11 (14.86)
Analgesic/anti-inflammatory	5 (15.62)	10 (27.77)	2 (66.6)	1 (100.0)	2 (100.0)	18 (24.32)
Diclofenac	0	3	1	0	0	4 (5.40)
Ibuprofen	0	3	0	0	0	3 (4.05)
Naproxen	0	1	0	0	1	2 (2.70)
Nimesulide	0	2	0	0	0	2 (2.70)
Tenoxican	0	1	0	0	0	1 (1.35)
Dipyrone	4	3	0	1	1	9 (12.16)
Acetominophen	1	0	1	0	0	2 (2.70)
Non-antimicrobial sulfonamides	5 (15.62)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	5 (6.75)
Dapsone	2	0	0	0	0	2 (3.07)
Sulfasalazine	2	0	0	0	0	2 (3.07)
Sulfadiazine	1	0	0	0	0	1 (1.35)
Dexamethasone	0	0	1	0	0	1 (1.35)

SJS: Stevens-Johnson Syndrome; TEN: Toxic Epidermal Necrolysis; DRESS: Drug Reaction with Eosinophilia and Systemic Symptoms; AGEP: Acute Generalized Exanthematous Pustulosis; GBFDE: Generalized Bullous Fixed Drug Eruption.

In this series, asymptomatic hyperuricemia was the main motivation for the treatment with allopurinol (80% of patients) in ALLO group. High doses of ALLO associated use of diuretics and renal failure increase SCAR risk besides genetic factors (29). As not all patients with hyperuricemia develop gout or renal disease, ALLO therapy for asymptomatic hyperuricemia should be discouraged (30). The 2012 American College of Rheumatology Guidelines for Management of Gout recommend HLA genotyping in selected subpopulations (individuals of Korean descent with stage 3 or worse chronic kidney disease and those of Han-Chinese or Thai descent) prior starting ALLO treatment (31).

In our DRESS/DIHS cases, clinical-laboratory findings were similar to those previously described in literature (32) and corticosteroids were the mainstay of treatment in all patients. IVIG was also used in 2 of them with good clinical evolution (33). Corticosteroids were used in the majority of patients in the SJS/TEN group. Although it is classically related to higher mortality rates (34), there were no deaths in this group, maybe due to the greater frequency of patients with cutaneous detachment < 10%, since the main cause of death is septic shock, which is directly related to the extent of detachment (35). Patients with AGEP, GBFDE and overlap DRESS/AGEP and DRESS/SJS also received corticotherapy, without complications.

Two patients died of DRESS. Although the most common cause of death n DRESS is liver failure (22) all patients in our series recovered liver function, except for a CBZ-DRESS patient who died of multiple organ failure. A vancomycin-DRESS patient died with suspected diagnosis of acute necrotizing eosinophilic myocarditis. He developed cardiogenic shock in the first week of disease. Cardiac involvement is a rare and potentially fatal complication that can range from up to 4 months or evolve to long-term heart failure (36). Milder forms (hypersensitivity myocarditis) are probably underdiagnosed due to self-limited nature, nonspecific symptoms and should always be tracked for their potential severity (37). Although endomyocardial biopsy is considered a gold standard, in view of the dramatic evolution and critical clinical status of our patient, the diagnosis was made only in clinical grounds.

DRESS has a prolonged course and clinical reactivations can occur during the acute phase of reaction related to sequential latent viral reactivations of *Herpesviridae* family (HHV6, HHV7, EBV and CMV), flare-up reaction after introduction of new drugs or immune reconstitution syndrome by decrease of corticotherapy (38-41). Although we cannot rule out the possibility of viral reactivation in the presented cases, since it was not possible to confirm them by serology or polymerase chain reaction, it is possible that tapering of corticotherapy was the main cause of clinical reactivations in some of them.

Considering all types of SCAR, AGEP presents a lower chance of complications since it generally does not evolve with systemic involvement and tends to resolve faster without complications. The observed mean of latency period was slightly higher than observed in other series. Although antibiotics are classically con-

Table III *- Drug Patch Test (SCAR).*

Case	Drug	SCAR	C (%)	T (years)	Results
1	**CBZ**	**DRESS**	**5**	**< 1**	**Positive**
2	**CBZ**	**DRESS**	**10**	**1-2**	**Positive**
3	**CBZ**	**DRESS**	**10**	**< 1**	**Positive**
4	**CBZ**	**DRESS**	**10**	**1-2**	**Positive**
5	**CBZ**	**DRESS**	**5**	**< 1**	**Positive**
6	**PB**	**DRESS**	**30**	**< 1**	**Positive**
7	CBZ	DRESS	5	1-2	Negative
8	PHT	DRESS	30	4-5	Negative
9	PHT	DRESS	10	3-4	Negative
10	SZ	DRESS	10**	3-4	Negative
11	DAP	DRESS	10**	1-2	Negative
12	CEF	DRESS	30	2-3	Negative
13	AC/DIP	DRESS	10**	5-6	Negative
14	ALLO	DRESS/AGEP	30	1-2	Negative
15	Multiple*	SJS/TEN	10	1-2	Negative
16	LMT	SJS/TEN	30	1-2	Negative
17	LMT	SJS/TEN	10**	1-2	Negative
18	PB	SJS/TEN	30	1-2	Negative
19	PB	SJS/TEN	30	2	Negative
20	PB	SJS/TEN	30	3-4	Negative
21	CBZ	SJS/TEN	30	2-3	Negative
22	CBZ	SJS/TEN	30	1-2	Negative
23	CBZ	SJS/TEN	30	3-4	Negative
24	CBZ	SJS/TEN	30	6-7	Negative
25	LEVO	SJS/TEN	10**	< 1	Negative
26	AZI/DIP	SJS/TEN	30/10**	< 1	Negatve
27	ALLO	SJS/TEN	10**	5-6	Negative
28	ALLO	SJS/TEN	10**	1-2	Negative
29	AMX/CT	AGEP	30/1	< 1	Negative
30	NSAID	AGEP	10**	< 1	Negative

AC: acetaminophen; ALLO: allopurinol; CBZ: carbamazepine; C: concentration; CEF: ceftriaxone AP: dapsone; DIP: dipyrone; PB: phenobarbital; PHT: phenytoin; SZ: sulfasalazine; Multiple*: (SMX:S ulfamethoxazole; NVP: nevirapine; LMT: lamotrigine; PHT: phenytoin; TNX: tenoxican); 10**: pure substance a 10%; SJS: Stevens-Johnson syndrome; TEN: toxic epidermal necrolysis; T: time; DRESS: drug reaction with eosinophilia and systemic symptoms; AGEP: acute generalized exanthematous pustulosis: GBFD: generalized bullous fixed drug eruption.

sidered the most common cause, this was the suspected cause in only one of our cases, which acute localized exanthematous pustulosis (ALEP) (42, 43).

The patient with a diagnosis of GBFDE initially had a diagnosis of SJS/TEN. However, the retrospective evaluation of the history of 3 episodes with additional and progressive involvement of other skin areas after repeated expositions suggested a reassessment of the diagnostic hypothesis. GBFDE is a common and frequent mimicker of SJS/TEN cases (34). Some clues such as short latency, less constitutional symptoms and less mucosal involvement help the differential diagnosis (35).

The pharmacogenetic approach in SCAR patients is based on scientific evidence linking genetic factors such as HLA alleles and/or polymorphisms in drug-metabolizing genes related to increased susceptibility to SCAR by specific drugs and phenotypes (44, 45). Based on cost-effectiveness studies, international regulatory agencies such as Food and Drug Administration (FDA) and the European Medicine Agency (EMA) included specific drug-label information on the utility of screening patients who are candidates for use of ABC (B*57:01) and CBZ (B*15:02), regardless of ethnicity in the former and in patients with southeast Asian origin in the latter (46). Although in some groups as Asian and European people, respectively for ALLO (B*58:01) and CBZ (A*31:01), studies of cost-effectiveness have already been delineated, there is no recommendation from that agencies for routine screening (47, 48). However, in the presence of at least one copy of the HLA risk allele related to ABC, ALLO or CBZ, their use should be avoided (49-51). Risk alleles such as HLA-A*31:01 (CBZ), B*57:01 (ABC) and B*58:01 (ALLO), were identified in this study and helped to reinforce causality in eight SCAR patients. According to updated data from the Brazilian Bone Marrow Donor Network (Portuguese acronym: REDOME), the estimated frequency of alleles in the Brazilian population shows variation between the different skin colour and skin race group from 2.1 to 4%, from 2.7 to 2.9%, and 4.5 to 5.3% respectively for the B*58, B*57 and A*31 alleles (52). In our study, the self-attributed skin color referred to ancestry up to great-grand parents was investigated. Although skin color is determined by several factors and its relation to ancestry is inconsistent, this has been the main form of stratification used in scientific studies (53, 54). The genetic diversity of the Brazilian population determined by centuries of interbreeding of races was confirmed in this group by the majority of the self-attributed brown color. It is emphasized that the distance of the historical events that characterized the miscegenation of the Brazilian population compromised the knowledge of the ancestral roots in most cases.

To evaluate the risk and benefit for the use of drugs highly implicated in SCAR, the pharmacogenetic evaluation of ABC, CBZ and ALLO in selected cases for both diagnostic evaluation and case prevention, besides other non-genetic risk factors like drug interactions and renal failure, is useful. The value of the routine genetic test and the possibility of new risk haplotypes are still unknown and there is a lack of cost-effectiveness studies in Brazilian population.

In vitro tests to confirm the culprit drug is limited by lack of validation. Lymphocyte transformation test (LTT), enzyme linked immunosorbent assay (ELISA) and enzyme-linked immunospot (ELISPOT) assay are available only at some research centers. Recently combined cytokine and cytotoxicity assays

Table IV *- HLA-A, -B and -DRB1 alleles in DRESS, SJS/TEN and DRESS/AGEP samples with HLA-A*31, -B*57 or -B*58.*

DRUG	SCAR	HLA-A*	HLA-B*	HLA-DRB1*
CBZ	DRESS	24:02:01:01	40:02:01:01	04:05:01:01
		31:01:02:01	51:01:01:01	04:05:01:03
CBZ	DRESS	**31:01:02:01**	39:03:01:01	13:02:01:01
		34:02:01	81:01:01	14:02:01:02
ABC	DRESS	01:01:01:01	38:01:01:01	04:02:01
		26:01:01:01	**57:01:01:01**	13:01:01:02
ALLO	SJS/TEN	02:01:01	**58:01:01**	11:02:01
		32:01:01	40:02:01	16:02:01
ALLO	SJS/TEN	23:17:01:01	42:02:01:02	07:01:01:01
		30:01:01:01	**58:01:01:01**	12:01:01:01
ALLO	SJS/TEN	03:01:01:01	35:01:01:05	03:01:01:01
		30:02:01:01	**58:01:01:01**	11:04:01
ALLO	DRESS/AGEP	02:02:01:01	53:01:01	13:02:01:03
		33:03:01:01	**58:01:01:03**	15:03:01:02
ALLO	SJS/TEN	01:01:01:01	35:01:01:05	01:01:01
		03:01:01:01	**58:01:01:01**	07:01:01:01

ALLO: Allopurinol; ABC: Abacavir; CBZ: Carbamazepine; AGEP: Acute Generalized Exanthematous Pustulosis; DRESS: Drug Reaction with Eosinophilia and Systemic Symptoms; SJS: Stevens-Johnson Syndrome; TEN: Toxic Epidermal Necrolysis. HLA Typing were performed with massive parallel sequencing.

(Cyto-LTT) has improved the sensitivity for identification of the drug in the resolution phase of SJS/TEN. They were not carried out in this study (55).

Skin tests also need validation and sensitivity varies with the drug, phenotype and the time since the reaction. Faced with the lack of knowledge about how long it can remain positive, it is suggested to perform them during the first year after resolution of SCAR to avoid false negative results (56, 57).

The value of DPT in DRESS has been described with sensitivity ranging from 32-70% (6, 58, 59). Regarding CBZ, the DPT sensitivity in patients with DRESS/DIHS ranged from 60 to 84.6% (59,6). It was confirmed in our study since five out of six CBZ-DRESS had positive DPT, besides one PB-DRESS. Considering all DPT applied in DRESS/DIHS in our cases, the sensitivity of the method was 42.85%, with sensitivity of 83.33% in DRESS/DIHS by CBZ, even in low concentrations (1-10%). The low sensitivity of DPT in SJS/TEN has been verified in most of published series, varying from 9-23% (6, 60). In present study, none of SJS/TEN patients, including CBZ-SJS/TEN cases had positive DPT, which confirms the low sensitivity of the method in this phenotype.

DPT are safe in the SCAR investigation, however test-induced reactivities are described especially for CBZ and acyclovir (56). In our series, one CBZ-induced DRESS patient presented rash and fever without organ involvement 24 hour after a negative 96 hour-reading DPT. Relapses in AGEP and DRESS have been described even though their DPT results were negative (6, 56). None of the other patients presented reactions related to DPT application. Given the impossibility of validation by a standard test, negative skin tests do not exclude the involvement of the suspected drug (61).

Restriction of involved drug groups were based on analysis of their culpability started at least eight weeks prior to the index reaction day. After application of causality algorithms, the drugs that showed a possible or defined score were defined as suspect, whether or not they were reinforced by DPT and/or genetic test. There are limitations in our study, such as the absence of a systematic biopsy in all patients, which is not necessary for clinical purposes, as well the non-accomplishment of *in vitro* methods. The use of a medium resolution method for HLA genotyping did not allow us the identification of four digits HLA alleles in all patients. It is known that patients tolerant to a particular drug may carry risk alleles for that same drug. However, the identification of alleles related to CBZ and ABC in patients with DRESS and ALLO in patients with SJS/TEN strongly reinforced causality and contraindicated their later use and additionally allowed pharmacogenetic guidance for patients and their relatives.

To our knowledge this is the first published case series of SCAR with documented HLA alleles and DPT in Brazil. It can be useful to other researchers and for clinical physicians working with SCAR patients in Brazil and in other middle-income countries. Moreover, multicentric studies in mixed Brazilian population may be helpful in the search for new related risk alleles. The high genetic admixture makes up a mosaic of genome ancestry with unique proportions in each Brazilian making the cost-effectiveness for genetic screening difficult. However, the possibility of

the existence of new haplotypes in an admixed population and their correlations with SCAR are still unknown and must be considered in the Brazilian population.

Conclusions

In conclusion, the systematic approach using tools for phenotyping and causality identification of SCAR are essential for the study of correlation with *in vitro* and *in vivo* methods including HLA typing and DPT. Despite the lack of validation, DPT were safe and effective to establish the diagnosis in CBZ-induced DRESS. Through HLA genotyping, the HLA-A*31:01, B*57:01 and B*58:01 alleles were identified, reinforcing its implication in SCAR induced by CBZ, ABC and ALLO, among the Brazilian population.

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Conflict of interests

The authors declare that they have no conflict of interests.

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Pre-treatment allergen-specific IgE analysis and outcomes of allergen immunotherapy

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Key words

Pollen allergy; IgE; allergen immunotherapy; birch; grass.

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Impact statement

No correlation exists between profile of allergen sensitization and effect of alleregn immunotherapy.

Summary

Background. *Patients show varied results to allergen immunotherapy (AIT). The reason for this variability is unclear.* ***Objective.*** *To describe the relationship between AIT efficacy and demographic characteristics, as well as pre-treatment plasma levels of specific IgE-antibodies to grass and birch pollen.* ***Methods.*** *A retrospective study was performed based on medical records of 128 patients who received AIT. The patients completed a questionnaire and pre-AIT plasma levels of allergen-specific IgE to grass and birch pollen were measured using EUROLINE DPA-Dx pollen 1 method.* ***Results.*** *Seventy percent of patients classified their allergic symptoms as less severe after AIT. Twenty-seven percent had received AIT targeting only grass pollen, 19% targeting only birch pollen, and 55% targeting both grass and birch. A total of 35 different IgE profiles were found across our study population. On comparison of the demographic characteristics and concentration of allergen-specific IgE-antibodies, no statistically significant differences could be found.* ***Conclusions.*** *The majority of patients rated their allergic symptoms as less severe after AIT. No clear relationship could be demonstrated between pre-treatment allergen-specific IgE concentration, or demographic characteristics, and effect of AIT. There may be other factors underlying the different responses to AIT.*

Introduction

Allergic rhinitis (AR) affects up to 30% of the world's population and therefore poses a great socioeconomic burden (1). Allergen immunotherapy (AIT) is the only disease-modifying treatment for Immunoglobulin E (IgE)-mediated allergic disease (2). In addition to being capable of modifying disease, it shows long-term effects after treatment is achieved; in other words, it is capable of curing allergies (3). The treatment is based on the administration of allergen extracts which are complex mixtures, with not all extracts having the same allergenic properties (4, 5). Some extracts may lack some allergenic proteins or may be impaired during the production process and storage (5). Moreover, the effectiveness of AIT can vary between patients (6, 7). Most patients are significantly improved after AIT, with a response rate of around 80% (8, 9). However, not all allergic individuals respond to AIT, and furthermore AIT is not as effective in treating hypersensitivity to all different allergens (2), and the reason for this is unclear. Furthermore, it has been shown that the effect decreases when treating for several allergens simultaneously (10). It is difficult to predict how patients will respond to the treatment, but many studies have tried to find suitable biomarkers to predict the clinical effect of AIT (11-14).

More research is needed to gain a better understanding of exactly why AIT does not work for all patients. This knowledge will in turn provide opportunities for establishing the optimal dose and method of administration (2). Diagnostic biomarkers help to select the patients who will be the best responders to a specific treatment (2). Analysis of allergen-specific IgE (sIgE) has been proposed as a biomarker for AIT (1). The use of allergen components is of great diagnostic importance to identify the main sensitizing component.

One of the aims of this study is to describe the relationship between AIT efficacy and demographic characteristics. Another aim is to study plasma levels of sIgE to grass and birch pollen prior to AIT, measured using the component-resolved, multiplex immunoblot test system, EUROIMMUNE (EUROIMMUN AG, Lübeck, Germany).

Materials and methods

Study design and population

This study is an observational, retrospective study on a cohort of patients with grass and/or birch pollen allergy who received AIT between 1999 and 2015 at the Otorhinolaryngology and Pulmonology Departments, Örebro University Hospital, Örebro, Sweden. Adult patients with a history of AR, positive skin prick test, and/or allergen-specific IgE test were included in the study. The study was approved by the Swedish Ethical Review Authority. Written consent to participate in the study was collected from all included patients.

After completion, the effectiveness of AIT was assessed by the patients who completed a questionnaire for evaluation of allergic symptoms both before and after receiving AIT, using a 10 cm numeric rating scale (NRS) ranging from 0 (no symptoms) to 10 (severe symptoms). For subjects who reported suffering from asthma, both AR and asthmatic symptoms were assessed together on one single NRS. The study subjects were stratified into non-responders and responders based on whether their AR and/or asthmatic symptoms had improved, *i.e.*, changed from severe symptoms before, to moderate or mild symptoms after AIT. The questionnaire contained questions about demographic characteristics, asthma, duration of the patient's allergic symptoms, what medication the patient used, degree of satisfaction with the treatment, change in quality of life, and whether the patient had suffered from any side effects from the treatment.

Skin prick test and immunotherapy

Products from ALK- Abelló (Hørsholm, Denmark) were used for the skin prick test and AIT. Soluprick SQ® was used for the skin prick test. For AIT against grass pollen allergy, the majority of patients (n = 101) received Alutard SQ® 5 Grasses, and only three patients received Alutard SQ® Timothy grass (*Phleum pratense*). The majority of patients with birch pollen allergy (n = 86) were treated with Alutard SQ® Birch (*Betula verucosa*) and only eight patients were treated with Alutard SQ® 3 Trees (*Betula verucosa*, *Alnus glutinosa*, and *Corylus avellana*). Subjects who were treated simultaneously for both grass and birch allergy received a combination of either Alutard SQ® 5 Grasses and Alutard SQ® Birch (n = 59), or Alutard SQ® 5 Grasses and Alutard SQ® 3 Trees (n = 8), or Alutard SQ® Timothy grass and Alutard SQ® Birch (n = 3).

Immunoglobulin E analysis

Serum IgE antibodies were measured using EUROBlotOne, EUROLINE DPA-Dx pollen 1 (EUROIMMUN AG, Lübeck, Germany), according to the manufacturer's instructions. The results were expressed in kU/L, with a cutoff value of 0.35 kU/L as a positive result. The test kit contained strips lined with parallel bands, for eleven different allergens, and a control band (indicator band). Serum samples were analyzed for specific IgE against eleven different allergens, *Betula verrucosa*, birch (t3), and the birch components rBet v 1, rBet v 2, rBet v 4, rBet v 6, and *Phleum pratense*, Timothy grass (g6), and the Timothy components rPhl p 1, rPhl p 5, rPhl p 7, rPhl p 12, and cross-reactive carbohydrate determinants (CCDs). A known control sample, positive for birch (t3) and Timothy (g6), was run with each analysis to ensure that the method worked as intended.

Statistics

Microsoft® Excel (Microsoft, Seattle, W.A., USA) was used to store the data and to create the tables presented in this study. Mann-Whitney U-tests were used to compare interval or ordinal level variables between groups. Pearson's chi-squared tests (or Fisher's exact test where expected cell counts were < 5 in the cross-tabulation) were used for categorical variables as well as for comparison of sIgE levels between the different groups. Alpha levels were set to 0.05 for all tests. All statistical analyses were performed using IBM SPSS Statistics for Windows, version 26 (IBM Corp., Armonk, N.Y., USA).

Results

Out of 353 patients who were contacted for participation in the study, 128 patients were included, as illustrated in **figure 1**. Thirty-four (27%) patients underwent AIT targeting only grass pollen, 24 (19%) targeting only birch pollen, and 70 (55%) targeting both grass and birch. Twelve (9%) patients also received treatment targeting an additional allergen other than grass and/or birch at the same time (seven were treated for pet allergy, four for mugwort, and one for house dust mite (HDM)). In total, 74 (58%) subjects were treated with at least two allergens simultaneously (62 with grass and birch; three with grass, birch, and mugwort; three with grass, birch, and cat hair; two with grass and cat hair; one with grass, birch, and HDM; one with grass,

birch, and dog hair; one with grass and mugwort; and one with grass, dog hair, and cat hair).

Ninety (70%) patients classified their AR and/or asthmatic symptoms as less severe after, compared to before, AIT, as shown in **figure 2**. There were no statistically significant differences between non-responders and responders regarding age, sex, prevalence of asthma, or prior pharmacological treatment in any of the three treatment groups. Complete characteristics of the patients, stratified by target allergen, are given in **table I**. Responders were more satisfied with their AIT compared to non-responders across the subgroups treated for either grass, or grass and birch (8.75 *vs* 5, p = 0.014; 9 *vs* 6, p = 0.021). The responders were more satisfied also in the subgroup of patients treated for only birch allergy; however, without a statistically significant difference (9 *vs* 7.75, p = 0.214). Across all three treatment groups, there were statistically significant differences in how non-responders and responders rated how much their quality of life was affected by their allergy after completed AIT (grass: 5.5 *vs* 2, p < 0.001; birch: 7 *vs* 2.25, p = 0.004; grass and birch: 6 *vs* 2, p = 0.001) (**table I**).

In the group of patients treated for both grass and birch allergy, a total of 18 different molecular patterns were observed, 17 of which were found in the patients treated only for grass allergy, and nine in those treated for birch allergy only (**figure 3**).

There were no large differences in molecular spread (defined as number of sIgE molecules to which a subject is sensitized) between non-responders and responders. However, within the subgroup of subjects treated for both grass and birch, the molecular spread was marginally lower in non-responders compared to responders (interquartile range (IQR) 4-5 *vs* IQR 5-6, p = 0.017) (**table II**).

There was a significant difference in dispersion of concentration of sIgE to g6 molecules within subjects treated for both grass and birch. However, no obvious linear relationship could be seen. On comparison of the concentration of allergen sIgE antibodies within the other treatment groups, no statistically significant differences were found (**table II**).

The levels of sIgE were also compared between non-responders and responders when stratified based on what type of symptoms the subjects experienced, AR only or AR and asthmatic (**table III**). There was only a statistically significant difference in dispersion of level of sIgE to g6 between non-responders and

Figure 1 *- Flowchart of the inclusion process.*

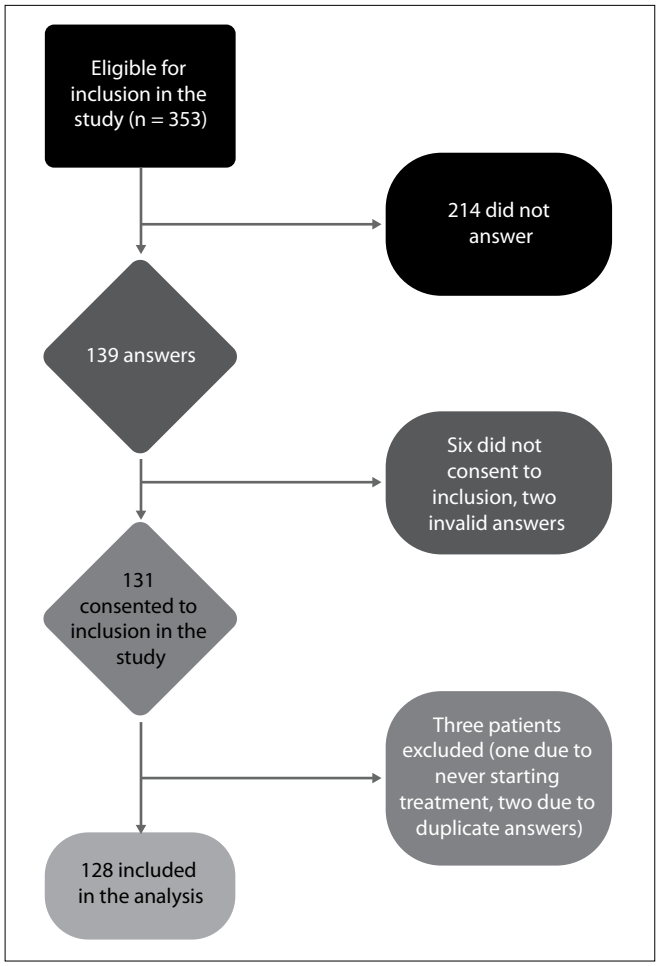
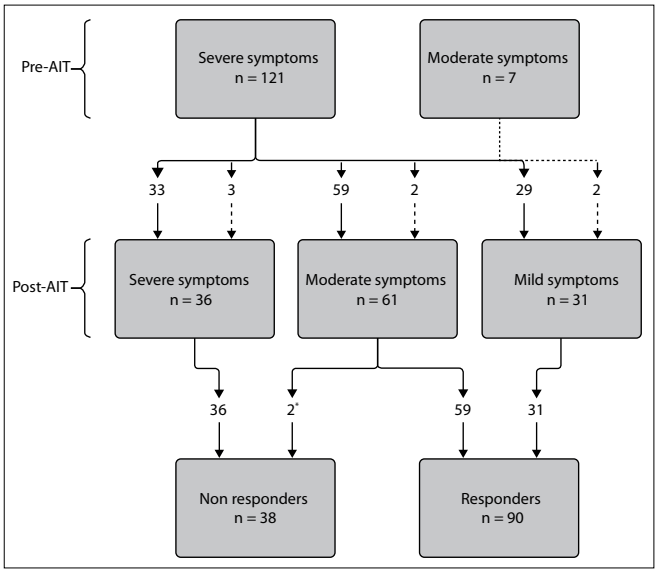


Figure 2 *- Pre- vs post-AIT symptom severity of the study subjects.*



AIT: Allergen ImmunoTherapy; *two patients rated their symptoms as moderate both before and after treatment, and thus are not considered as improved.

Table I *- Characteristics of the study subjects both total and stratified by treatment groups.*

		Total			Grass			Birch			Grass and birch		
		Non-responders	**Responders**	**P-value**	**Non- responders**	**Responders**	**P-value**	**Non- responders**	**Responders**	**P-value**	**Non- responders**	**Responders**	**P-value**
N		38	90	–	12	22	–	8	16	–	18	52	–
Age, yrs	median (min–max)	32 (20–40.5)	32.5 (24.75–40)	0.518	37.5 (19.5–42.75)	37.5 (28.25–43.5)	0.631	36.5 (28.5–44.5)	35 (26–40)	0.610	25.5 (19.75–35.5)	29.5 (24–36.75)	0.160
	< 30	18 (47%)	38 (42%)	0.592	4 (33%)	6 (27%)	0.714	2 (25%)	6 (38%)	0.667	12 (67%)	26 (50%)	0.221
Sex	Female	26 (68%)	51 (57%)	0.215	7 (58%)	12 (55%)	0.832	6 (75%)	13 (81%)	1.000	13 (72%)	26 (50%)	0.102
Additional target allergen		3 (8%)	9 (10%)	1.000	2 (17%)	2 (9%)	0.602	0 (0%)	0 (0%)	–	1 (6%)	7 (13%)	0.670
Pre-AIT treatment	Antihistamines	34 (89%)	80 (89%)	1.000	11 (92%)	21 (95%)	1.000	7 (88%)	15 (94%)	1.000	16 (89%)	44 (85%)	1.000
	Local nasal steroids	31 (82%)	61 (68%)	0.113	9 (75%)	16 (73%)	1.000	7 (88%)	10 (63%)	0.352	15 (83%)	35 (67%)	0.195
	Systemic steroids	11 (29%)	31 (34%)	0.545	3 (25%)	5 (23%)	1.000	3 (38%)	5 (31%)	1.000	5 (28%)	21 (40%)	0.340
Asthma	Diagnosed	10 (26%)	24 (27%)	0.967	2 (17%)	5 (23%)	1.000	3 (38%)	7 (44%)	1.000	5 (28%)	12 (23%)	0.753
	Self-reported	19 (50%)	42 (47%)	0.730	5 (42%)	9 (41%)	1.000	5 (63%)	10 (63%)	1.000	9 (50%)	23 (44%)	0.672
Pre-AIT asthma treatment**	Bronchodilator	11 (65%)	34 (72%)	0.555	4 (80%)	9 (90%)	1.000	3 (75%)	7 (64%)	1.000	4 (50%)	18 (69%)	0.410
	ICS	9 (53%)	30 (64%)	0.430	3 (60%)	8 (80%)	0.560	2 (50%)	8 (73%)	0.560	4 (50%)	14 (54%)	1.000
	LTRA	2 (12%)	2 (4%)	0.285	0 (0%)	1 (10%)	1.000	0 (0%)	0 (0%)	-	2 (25%)	1 (4%)	0.131
	None	5 (29%)	9 (19%)	0.495	1 (20%)	0 (0%)	0.333	1 (25%)	3 (27%)	1.000	3 (38%)	6 (23%)	0.649
Satisfaction with treatment		6.5 (0.5–10)	9 (0–10)	< 0.001*	5 (1–10)	8.75 (1–10)	0.014*	7.75 (0.5–10)	9 (3–10)	0.214	6 (2.5–10)	9 (0–10)	0.021
QoL after treatment		6 (1–10)	2 (0–10)	< 0.001*	5.5 (1–8)	2 (0–4)	< 0.001*	7 (1.5–10)	2.25 (0–5)	0.004*	6 (1–9)	2 (0–10)	< 0.001*

Categorical variables are presented as frequency (percentage), interval and ordinal level variables as median (IQR). P-values presented are from $\chi 2$ or Fisher's exact-tests for categorical variables, and Mann Whitney U-tests for interval and ordinal variables. AIT: Allergen Immunotherapy; ICS: Inhaled Corticosteroids; LTRA: Leukotriene Receptor Antagonists; QoL: Quality of Life; *P-value < 0.05; **only reported for subjects with asthma according to medical records and/or questionnaire.

Table II *- Comparison of molecular spread and specific Immunoglobulin E (IgE) levels between improved and non-improved subjects, stratified by treatment group.*

		Total			Grass			Birch			Grass and birch		
		Non-responders	Responders	P-value	Non-responders	Responders	P-value	Non-responders	Responders	P-value	Non-responders	Responders	P-value
N		38	90	–	12	22	–	8	16	–	18	52	–
Molecular spread		4 (3.75–5)	5 (3–6)	0.097	4 (3–4.75)	4 (3–5.25)	0.929	2 (2–5)	3 (2–4)	0.928	5 (4–5)	5 (5–6)	0.017*
p12	< 0.35	33 (87%)	76 (84%)	0.710	9 (75%)	18 (82%)	0.156	7 (88%)	15 (94%)	0.565	17 (94%)	43 (83%)	0.141
	0.35–3.4	2 (5%)	6 (7%)		2 (17%)	0 (0%)		0 (0%)	0 (0%)		0 (0%)	6 (12%)	
	3.5–49.9	1 (3%)	6 (7%)		0 (0%)	3 (14%)		1 (13%)	0 (0%)		0 (0%)	3 (6%)	
	> 50	2 (5%)	2 (2%)		1 (8%)	1 (5%)		0 (0%)	1 (6%)		1 (6%)	0 (0%)	
p7	< 0.35	37 (97%)	88 (98%)	0.359	12 (100%)	21 (95%)	1.000	7 (88%)	16 (100%)	0.333	18 (100%)	51 (98%)	1.000
	0.35–3.4	1 (3%)	0 (0%)		0 (0%)	0 (0%)		1 (13%)	0 (0%)		0 (0%)	0 (0%)	
	3.5–49.9	0 (0%)	0 (0%)		0 (0%)	0 (0%)		0 (0%)	0 (0%)		0 (0%)	0 (0%)	
	> 50	0 (0%)	2 (2%)		0 (0%)	1 (5%)		0 (0%)	0 (0%)		0 (0%)	1 (2%)	
p5	< 0.35	14 (37%)	26 (29%)	0.124	1 (8%)	4 (18%)	0.866	6 (75%)	14 (88%)	0.196	7 (39%)	8 (15%)	0.077
	0.35–3.4	3 (8%)	1 (1%)		0 (0%)	0 (0%)		2 (25%)	0 (0%)		1 (6%)	1 (2%)	
	3.5–49.9	4 (11%)	9 (10%)		2 (17%)	3 (14%)		0 (0%)	1 (6%)		2 (11%)	5 (10%)	
	> 50	17 (45%)	54 (60%)		9 (75%)	15 (68%)		0 (0%)	1 (6%)		8 (44%)	38 (73%)	
p1	< 0.35	5 (13%)	16 (18%)	0.126	0 (0%)	1 (5%)	1.000	5 (63%)	10 (63%)	0.178	0 (0%)	5 (10%)	0.283
	0.35–3.4	0 (0%)	4 (4%)		0 (0%)	0 (0%)		0 (0%)	3 (19%)		0 (0%)	1 (2%)	
	3.5–49.9	5 (13%)	3 (3%)		0 (0%)	0 (0%)		2 (25%)	0 (0%)		3 (17%)	3 (6%)	
	> 50	28 (74%)	67 (74%)		12 (100%)	21 (95%)		1 (13%)	3 (19%)		15 (83%)	43 (83%)	
g6	< 0.35	5 (13%)	12 (13%)	0.029*	0 (0%)	0 (0%)	0.537	5 (63%)	10 (63%)	0.115	0 (0%)	2 (4%)	0.004*
	0.35–3.4	0 (0%)	6 (7%)		0 (0%)	0 (0%)		0 (0%)	4 (25%)		0 (0%)	2 (4%)	
	3.5–49.9	9 (24%)	6 (7%)		0 (0%)	3 (14%)		2 (25%)	0 (0%)		7 (39%)	3 (6%)	
	> 50	24 (63%)	66 (73%)		12 (100%)	19 (86%)		1 (13%)	2 (13%)		11 (61%)	45 (87%)	
v6	< 0.35	36 (95%)	82 (91%)	0.129	11 (92%)	22 (100%)	0.353	8 (100%)	14 (88%)	1.000	17 (94%)	46 (88%)	0.249
	0.35–3.4	0 (0%)	6 (7%)		0 (0%)	0 (0%)		0 (0%)	1 (6%)		0 (0%)	5 (10%)	
	3.5–49.9	1 (3%)	2 (2%)		0 (0%)	0 (0%)		0 (0%)	1 (6%)		1 (6%)	1 (2%)	
	> 50	1 (3%)	0 (0%)		1 (8%)	0 (0%)		0 (0%)	0 (0%)		0 (0%)	0 (0%)	
v4	< 0.35	38 (100%)	88 (98%)	1.000	12 (100%)	21 (95%)	1.000	8 (100%)	16 (100%)	–	18 (100%)	51 (98%)	1.000
	0.35–3.4	0 (0%)	1 (1%)		0 (0%)	1 (5%)		0 (0%)	0 (0%)		0 (0%)	0 (0%)	
	3.5–49.9	0 (0%)	0 (0%)		0 (0%)	0 (0%)		0 (0%)	0 (0%)		0 (0%)	0 (0%)	
	> 50	0 (0%)	1 (1%)		0 (0%)	0 (0%)		0 (0%)	0 (0%)		0 (0%)	1 (2%)	
v2	< 0.35	33 (87%)	76 (84%)	1.000	8 (67%)	17 (77%)	0.726	8 (100%)	16 (100%)	–	17 (94%)	43 (83%)	0.299
	0.35–3.4	2 (5%)	7 (8%)		2 (17%)	2 (9%)		0 (0%)	0 (0%)		0 (0%)	5 (10%)	
	3.5–49.9	2 (5%)	5 (6%)		2 (17%)	2 (9%)		0 (0%)	0 (0%)		0 (0%)	3 (6%)	
	> 50	1 (3%)	2 (2%)		0 (0%)	1 (5%)		0 (0%)	0 (0%)		1 (6%)	1 (2%)	

		Total			Grass			Birch			Grass and birch		
		Non-responders	Responders	P-value	Non-responders	Responders	P-value	Non-responders	Responders	P-value	Non-responders	Responders	P-value
v1	< 0.35	10 (26%)	10 (11%)	0.203	9 (75%)	10 (45%)	0.428	0 (0%)	0 (0%)	1.000	1 (6%)	0 (0%)	0.514
	0.35–3.4	1 (3%)	5 (6%)		1 (8%)	4 (18%)		0 (0%)	0 (0%)		0 (0%)	1 (2%)	
	3.5–49.9	4 (11%)	13 (14%)		1 (8%)	4 (18%)		1 (13%)	1 (6%)		2 (11%)	8 (15%)	
	> 50	23 (61%)	62 (69%)		1 (8%)	4 (18%)		7 (88%)	15 (94%)		15 (83%)	43 (83%)	
t3	< 0.35	10 (26%)	13 (14%)	0.433	10 (83%)	13 (59%)	0.562	0 (0%)	0 (0%)	1.000	0 (0%)	0 (0%)	1.000
	0.35–3.4	2 (5%)	4 (4%)		1 (8%)	2 (9%)		0 (0%)	0 (0%)		1 (6%)	2 (4%)	
	3.5–49.9	4 (11%)	13 (14%)		0 (0%)	3 (14%)		1 (13%)	1 (6%)		3 (17%)	9 (17%)	
	> 50	22 (58%)	60 (67%)		1 (8%)	4 (18%)		7 (88%)	15 (94%)		14 (78%)	41 (79%)	
CCD	< 0.35	35 (92%)	76 (84%)	0.526	11 (92%)	19 (86%)	1.000	7 (88%)	16 (100%)	0.333	17 (94%)	41 (79%)	0.338
	0.35–3.4	1 (3%)	8 (9%)		1 (8%)	2 (9%)		0 (0%)	0 (0%)		0 (0%)	6 (12%)	
	3.5–49.9	2 (5%)	6 (7%)		0 (0%)	1 (5%)		1 (13%)	0 (0%)		1 (6%)	5 (10%)	
	> 50	0 (0%)	0 (0%)		0 (0%)	0 (0%)		0 (0%)	0 (0%)		0 (0%)	0 (0%)	

Molecular spread is presented as median (interquartile range (IQR)) with P-values from Mann-Whitney U-test. Allergen-specific IgE levels are presented as frequency (%), with P-values from Fisher's exact test. CCD: cross-reactive carbohydrate determinant; *P-value < 0.05.

Figure 3 *- Specific Immunoglobulin-E profiles for the subjects, stratified by target allergen.*

responders in the subgroup of patients who reported both AR and asthmatic symptoms.

Discussion

We found that 70% of the patients ranked their symptoms as less severe after completion of, compared to before, AIT (65%, 67%, and 74% for the subgroups treated for grass, birch, and both grass and birch, respectively). Despite difficulties in comparing studies due to differences in outcome measures, our findings correspond relatively well to earlier studies, which found response rates to AIT of around 80% (8, 9). This indicates that, although

Table III *- Comparison of specific Immunoglobulin E (IgE) levels between improved and non-improved subjects, stratified by symptom groups both as gathered from the subjects' medical records as well as according to the questionnaires.*

		Symptoms according to medical records						Symptoms according to questionnaire					
		AR only			AR and asthma			AR only			AR and asthma		
		Non-responders	Responders	P-value	Non-responders	Responders	P-value	Non-responders	Responders	P-value	Non-responders	Responders	P-value
N		28	66		10	24		19	48		19	42	
p12	< 0.35	26 (93%)	56 (85%)	0.521	7 (70%)	20 (83%)	0.690	18 (95%)	39 (81%)	0.247	15 (79%)	37 (88%)	0.648
	0.35-3.4	1 (4%)	4 (6%)		1 (10%)	2 (8%)		0 (0%)	4 (8%)		2 (11%)	2 (5%)	
	3.5-49.9	0 (0%)	5 (8%)		1 (10%)	1 (4%)		0 (0%)	4 (8%)		1 (5%)	2 (5%)	
	> 50	1 (4%)	1 (2%)		1 (10%)	1 (4%)		1 (5%)	1 (2%)		1 (5%)	1 (2%)	
p7	< 0.35	28 (100%)	65 (98%)	1.000	9 (90%)	23 (96%)	0.508	19 (100%)	47 (98%)	1.000	18 (95%)	41 (98%)	0.530
	0.35-3.4	0 (0%)	0 (0%)		1 (10%)	0 (0%)		0 (0%)	0 (0%)		1 (5%)	0 (0%)	
	3.5-49.9	0 (0%)	0 (0%)		0 (0%)	0 (0%)		0 (0%)	0 (0%)		0 (0%)	0 (0%)	
	> 50	0 (0%)	1 (2%)		0 (0%)	1 (4%)		0 (0%)	1 (2%)		0 (0%)	1 (2%)	
p5	< 0.35	9 (32%)	17 (26%)	0.561	5 (50%)	9 (38%)	0.115	7 (37%)	10 (21%)	0.379	7 (37%)	16 (38%)	0.085
	0.35-3.4	1 (4%)	1 (2%)		2 (20%)	0 (0%)		0 (0%)	1 (2%)		3 (16%)	0 (0%)	
	3.5-49.9	4 (14%)	7 (11%)		0 (0%)	2 (8%)		3 (16%)	5 (10%)		1 (5%)	4 (10%)	
	> 50	14 (50%)	41 (62%)		3 (30%)	13 (54%)		9 (47%)	32 (67%)		8 (42%)	22 (52%)	
p1	< 0.35	4 (14%)	10 (15%)	0.615	1 (10%)	6 (25%)	0.180	3 (16%)	7 (15%)	0.533	2 (11%)	9 (21%)	0.284
	0.35-3.4	0 (0%)	2 (3%)		0 (0%)	2 (8%)		0 (0%)	1 (2%)		0 (0%)	3 (7%)	
	3.5-49.9	3 (11%)	3 (5%)		2 (20%)	0 (0%)		2 (11%)	1 (2%)		3 (16%)	2 (5%)	
	> 50	21 (75%)	51 (77%)		7 (70%)	16 (67%)		14 (74%)	39 (81%)		14 (74%)	28 (67%)	
g6	< 0.35	4 (14%)	9 (14%)	0.424	1 (10%)	3 (13%)	0.041*	3 (16%)	5 (10%)	0.647	2 (11%)	7 (17%)	0.022*
	0.35-3.4	0 (0%)	2 (3%)		0 (0%)	4 (17%)		0 (0%)	1 (2%)		0 (0%)	5 (12%)	
	3.5-49.9	5 (18%)	5 (8%)		4 (40%)	1 (4%)		3 (16%)	4 (8%)		6 (32%)	2 (5%)	
	> 50	19 (68%)	50 (76%)		5 (50%)	16 (67%)		13 (68%)	38 (79%)		11 (58%)	28 (67%)	
v6	< 0.35	26 (93%)	62 (94%)	0.085	10 (100%)	20 (83%)	1.000	17 (89%)	43 (90%)	0.198	19 (100%)	39 (93%)	1.000
	0.35-3.4	0 (0%)	4 (6%)		0 (0%)	2 (8%)		0 (0%)	4 (8%)		0 (0%)	2 (5%)	
	3.5-49.9	1 (4%)	0 (0%)		0 (0%)	2 (8%)		1 (5%)	1 (2%)		0 (0%)	1 (2%)	
	> 50	1 (4%)	0 (0%)		0 (0%)	0 (0%)		1 (5%)	0 (0%)		0 (0%)	0 (0%)	
v4	< 0.35	28 (100%)	65 (98%)	1.000	10 (100%)	23 (96%)	1.000	19 (100%)	47 (98%)	1.000	19 (100%)	41 (98%)	1.000
	0.35-3.4	0 (0%)	1 (2%)		0 (0%)	0 (0%)		0 (0%)	1 (2%)		0 (0%)	0 (0%)	
	3.5-49.9	0 (0%)	0 (0%)		0 (0%)	0 (0%)		0 (0%)	0 (0%)		0 (0%)	0 (0%)	
	> 50	0 (0%)	0 (0%)		0 (0%)	1 (4%)		0 (0%)	0 (0%)		0 (0%)	1 (2%)	
v2	< 0.35	25 (89%)	56 (85%)	1.000	8 (80%)	20 (83%)	0.584	17 (89%)	41 (85%)	0.512	16 (84%)	35 (83%)	0.298
	0.35-3.4	2 (7%)	5 (8%)		0 (0%)	2 (8%)		2 (11%)	3 (6%)		0 (0%)	4 (10%)	
	3.5-49.9	1 (4%)	4 (6%)		1 (10%)	1 (4%)		0 (0%)	4 (8%)		2 (11%)	1 (2%)	
	> 50	0 (0%)	1 (2%)		1 (10%)	1 (4%)		0 (0%)	0 (0%)		1 (5%)	2 (5%)	

		Symptoms according to medical records						Symptoms according to questionnaire					
		AR only			AR and asthma			AR only			AR and asthma		
		Non-responders	Responders	P-value	Non-responders	Responders	P-value	Non-responders	Responders	P-value	Non-responders	Responders	P-value
v1	< 0.35	8 (29%)	9 (14%)	0.260	2 (20%)	1 (4%)	0.267	5 (26%)	8 (17%)	0.761	5 (26%)	2 (5%)	0.092
	0.35-3.4	0 (0%)	3 (5%)		1 (10%)	2 (8%)		0 (0%)	2 (4%)		1 (5%)	3 (7%)	
	3.5-49.9	3 (11%)	12 (18%)		1 (10%)	1 (4%)		3 (16%)	10 (21%)		1 (5%)	3 (7%)	
	> 50	17 (61%)	42 (64%)		6 (60%)	20 (83%)		11 (58%)	28 (58%)		12 (63%)	34 (81%)	
t3	< 0.35	8 (29%)	11 (17%)	0.639	2 (20%)	2 (8%)	0.699	5 (26%)	9 (19%)	0.940	5 (26%)	4 (10%)	0.282
	0.35-3.4	1 (4%)	3 (5%)		1 (10%)	1 (4%)		1 (5%)	3 (6%)		1 (5%)	1 (2%)	
	3.5-49.9	3 (11%)	10 (15%)		1 (10%)	3 (13%)		3 (16%)	8 (17%)		1 (5%)	5 (12%)	
	> 50	16 (57%)	42 (64%)		6 (60%)	18 (75%)		10 (53%)	28 (58%)		12 (63%)	32 (76%)	
CCD	< 0.35	27 (96%)	57 (86%)	0.443	8 (80%)	19 (79%)	0.821	18 (95%)	39 (81%)	0.462	17 (89%)	37 (88%)	0.453
	0.35-3.4	1 (4%)	6 (9%)		0 (0%)	2 (8%)		1 (5%)	5 (10%)		0 (0%)	3 (7%)	
	3.5-49.9	0 (0%)	3 (5%)		2 (20%)	3 (13%)		0 (0%)	4 (8%)		2 (11%)	2 (5%)	
	> 50	0 (0%)	0 (0%)		0 (0%)	0 (0%)		0 (0%)	0 (0%)		0 (0%)	0 (0%)	

Allergen-specific IgE levels are presented as frequency (%), with P-values from Fisher's exact test. CCD: cross-reactive carbohydrate determinant; AR: allergic rhinitis; *P-value < 0.05.

most patients did benefit from AIT, 30% of the study subjects underwent a time-consuming and resource-intensive treatment without any obvious positive clinical effect. Although these proportions might change if using more refined outcome measures, this highlights the importance of finding suitable predictive factors to better select the patients who will benefit from AIT.

In this study, the effect of AIT was assessed by a questionnaire using an NRS for the evaluation of allergic symptoms both before and after AIT. Although the NRS has not been validated for measuring the severity of AR, a similar instrument, the visual analog scale, has been shown to correspond well to AR severity (15) and evaluation of improvement in symptoms after AIT (16). However, a less subjective measure of symptoms, as well the inclusion of a control group, would be required to accurately measure the effect size of AIT treatment in individual patients.

Our study included several patients aged 50 years or older, but the median age of the subjects was 32 years, which could indicate that the target population for AIT is of a relatively young age. However, AIT can be considered for the treatment of AR despite old age if no other contraindications exist (17).

In this study, 60% of the subjects were female. This distribution may have had some effect on our results as it has been shown that, despite the same immunological mechanisms of allergy, there is a clear clinical difference between female and male allergic patients. From adolescence onwards, female subjects suffer more often from allergies. This difference points to a role of sex hormones, intake of contraceptives, pregnancy, and hormone replacement therapy (18). However, sex did not seem to have a significant influence on the effect of AIT in our findings.

Most of the subjects in this study were using antihistamines and local nasal steroids prior to receiving AIT, but a much smaller proportion of the subjects had been using systemic steroids. The proportion of patients who had used systemic steroids prior to AIT was slightly higher in the group who improved after AIT. Despite the lack of statistical significance, it is possible that use of systemic steroids could indicate a more severe form of allergy and support the current clinical practice of using AIT as a last line of treatment when conventional pharmacological treatment has failed (19).

Our findings showed that, across all subgroups, responders were more satisfied with their AIT compared to non-responders. Both patient-related factors such as age, and AIT-related factors such as duration and side effects, influence patients' satisfaction (20). We also found statistically significant differences in how patients in all subgroups rated their quality of life after completing AIT. This finding is well in accordance with reports by other researchers (21).

Serum IgE antibodies for birch and Timothy grass pollen were analyzed using the EUROLINE DPA-Dx pollen 1 method (EUROBlotOne, EUROIMMUN AG, Lübeck, Germany). The relevance of this method has been demonstrated (22).

Our results revealed a total of 35 different sIgE profiles across our 128 study subjects, which indicates the immense immunological heterogeneity in subjects who are allergic to the same pollen. Cipriani *et al.* and Tripodi *et al.* have shown that the number of these profiles varies greatly in different studies (16, 23). In our results, IgE profiles with a higher number of molecules (higher molecular spread) seemed to respond better to AIT targeting grass and birch simultaneously. However, it is important to note that no correction was made for multiple testing and, consequently, care should be taken when interpreting these P-values.

Our results revealed that Bet v 1 is the predominant birch pollen component, while Bet v 2, Bet v 4 and Bet v 6 are not common in the studied population. This finding confirms previously published data on sensitisation to these molecules in European populations (24).

Serum IgE to CCD was found in only 13% of the patients. This finding shows that most of the patients were not sensitive to this molecule, and there were no significant differences among the groups. Similarly, we could not find evidence that sIgE to panallergen molecules was more prevalent in subjects who responded to AIT than in those who did not. We could not show evidence of statistically significant differences in the levels of sIgE to panallergens (Phl p 7, Phl p 12, Bet v 2 and Bet v 4) among subjects who responded to AIT than in those who did not.

Moreover, from the outcome of this study no correlation could be seen between molecular sensitization profile for Phl p 7 and asthma. This is in contrast to what have been proposed that molecular sensitization to Phl p 7 is a reliable biomarker of asthma (25). From our results, no clear association could be found between pre-AIT concentration of sIgE and AIT outcome. Using IgE as a biomarker in AIT has been reported to have conflicting outcomes (16). One study found that a cutoff sIgE level >10 kU/L can be associated with perception of effective AIT (26). In our study, there were no similar cutoffs.

We did find a statistically significant difference in levels of sIgE to g6 in the subgroup of subjects treated for grass and birch, where there was a higher percentage of subjects in the responder group with sIgE to g6 > 50 kU/L. This could indicate that the concentration of sIgE to g6 shows promise as a predictor for AIT outcome. However, this does not hold true in the subgroups treated for grass or birch separately. When the subjects were stratified based on type of symptoms, this difference in g6 level between non-responders and responders were only statistically significant within the subjects who reported both AR and asthmatic symptoms.

Overall, we expected to see more distinct differences in the levels of the allergen-specific IgE for birch and grass components between subjects who responded to AIT and subjects who did not. The lack of statistically significant differences between responders and non-responders could indicate that there are other factors, not considered in this study, that dictate how well a patient will respond to AIT. An interesting yet unanswered question is whether sensitization profiles affect individual outcomes of AIT. It is not clear whether patients with different sensitization profiles respond differently to the same AIT (25). Although there is significant interest in patient-tailored AIT, no such product has yet reached the commercial market.

A factor that may have influenced our results is related to which specific allergen components are found in the aluminium-adsorbed extract (Alutard SQ® products, ALK-Abelló). Alutard SQ products contain several different pollen proteins, both the main allergen and most minor allergens from grass or birch, but the exact ratios of these are unknown. As the European regulation of allergen products allows for great variation, different batches of the Alutard SQ product can have different allergenic content (27). The standardization of allergen extract for diagnosis and therapy is still an open issue in allergology (25). It is possible that the exact constituents of Alutard SQ vary with the variability in pollen intensity in different years, which could influence the efficacy of AIT. It is therefore possible that the subjects who did not respond or who reported only a moderate effect after AIT were sensitized to an allergen component that was not found in the allergen extract used in the AIT. One weakness of our study is that not all subjects were treated with the same allergen mixture, as some were treated with single allergen mixtures and some with compound mixtures containing allergens from several different grass/tree species. However, the proportion of patients who received a different allergen mixture was so small that we do not believe it has affected our results.

An environmental factor which may have affected the levels of IgE in our study subjects is when the samples were collected, as the blood samples in this study were collected a few months before the start of the actual pollen season.

The main limitation of this study is the low response rate to the patient questionnaires. The risk of non-response bias must be considered, as patients who had a positive experience of their AIT may have been more inclined to answer the questionnaire, and vice versa. We cannot exclude that the outcomes may have been different if more patients had participated in the study. However, our results on the overall effect of AIT are relatively equal to the results of previous, similar studies, which may indicate that the sample is representative (9, 28).

Another limitation is the time elapsed from end of treatment to follow-up, since all patients answered the questionnaire in late 2018 or early 2019 while the time of the end of treatment varied from 2002 to 2018. Consequently, there is the possibility of recall bias, and this may eventually have affected the low response rate. In future research, it might be possible to include a correction for time to follow-up in the analysis to avoid such limitation. Another limitation could be the long time the blood samples were stored, as this can affect the composition and quality of the biomolecules (29).

Conclusions

We found that the majority of the patients rated their allergic symptoms as less severe after, compared to before, AIT. No clear relationship was demonstrated between pre-treatment sIgE concentration or demographic factors, and effect of AIT. As the patients who did not respond to treatment had the same phenotype and IgE profiles as those who responded, this clearly indicates that there may be other factors underlying the different treatment responses. This urges us to conduct further studies to look for other substances (biomarkers) that predict or have an effect on the outcome of AIT.

Conflict of interests

No potential conflict of interest was reported by the authors.

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Prescribing patterns of medication for respiratory diseases: cluster analysis of the Portuguese electronic prescription database

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Key words

Asthma; chronic obstructive pulmonary disease; cluster analysis; electronic prescribing; retrospective studies.

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Summary

We aimed to describe, for the first time, the prescribing patterns among patients on persistent respiratory treatment, from the Portuguese electronic prescription and dispensing database. This was a one-year retrospective population-based analysis of prescriptions (n = 39810) for medication for respiratory diseases and exacerbations. Cluster analysis was applied based on medication and prescribers' specialty. Prescribing patterns were grouped and labelled as: possible medication for asthma and allergic rhinitis (General Practitioners-GPs and allergists to younger patients); COPD (GPs and pulmonologists to older patients); asthma or Asthma-COPD Overlap (GPs and pulmonologists); exacerbation, infection and relievers. This analysis was an important first step to understand the Portuguese reality on the treatment of respiratory diseases.

Impact statement

Eleven different prescription patterns were revealed by unsupervised analysis of prescriptions for respiratory diseases and exacerbations - from the Portuguese electronic prescription and dispensing database - providing a new understanding of the Portuguese reality on the treatment of respiratory diseases.

Introduction

The goals of asthma and chronic obstructive pulmonary disease (COPD) management are to reduce symptoms and minimize the risk of future exacerbations, obtained by continuous assessment, treatment, and review of the patient's response (1, 2). Asthma and COPD are heterogeneous diseases with similarities in symptoms and management options, moreover, some patients present an overlap of asthma and COPD features (asthma-COPD overlap - ACO). Although the use of the term ACO is controversial and both its concept and terminology are not robust, it is useful in clinical practice when patients cannot be clearly classified into asthma or COPD (1).

Real-world data (RWD) routinely collected in the course of healthcare delivery (3) have an important role in acknowledging the use and effects of treatments, and the overall heterogeneity of chronic diseases (4). RWD has also been used to describe medication prescribing for asthma and DPOC (5–7).

For the analysis of RWD, the unsupervised statistical techniques are increasingly popular approaches to identify and reveal new insights among healthcare data (8). They aim to reveal possible natural clusters grouped by similar characteristics, otherwise not be apparent, in other words, not defined *a priori*. Each cluster should be as homogenous as possible and have minimal overlapping to the other

clusters. Common clustering methods are hierarchical, partitional and two-step (distance-based methods) and latent class analysis (model-based methods) (9). Unsupervised clustering methods have been used to reveal phenotypes of asthma (10, 11), COPD (12) and allergic diseases (13, 14), and to identify factors of increased healthcare utilization (15) and prescription patterns (16).

In Portugal the research based on RWD, namely based on the national electronic prescription database is scarce. Recently we reported an analysis of data from the Portuguese electronic prescription and dispensing database that showed an association between insufficient prescription of maintenance medication and over-prescription of short-acting beta2 agonists (SABA) and oral corticosteroids (OCS) (17). Further research on maintenance prescription patterns may contribute to a better understanding of the underlying challenges of the management of chronic respiratory diseases in "real-world" healthcare.

Aims

We aim to describe medication patterns in the Portuguese electronic prescription and dispensing database (Portuguese electronic prescription and dispensing database (*Base de Dados Nacional de Prescrições*) - BDNP), among patients over 15 years old with persistent respiratory treatment (PRT).

Methods

Study design

This study was a retrospective population-based analysis of a random sample of patients from the Portuguese electronic prescription and dispensing database (BDNP).

Setting/Data source

The BDNP records data of all the prescriptions and respective dispensing in mainland Portugal. The population of interest in this study consists of patients to whom medication for respiratory and/or allergic diseases and exacerbations was prescribed at least once, between January 2016 and December 2016. We obtained all the prescriptions from a random sample of 2% (n = 103647) of these patients, corresponding to 1129512 prescriptions (**figure 1**). A more detailed description of the data source has been previously published (17).

Participants

In this study, we analysed the prescriptions (n = 248045) between January 2016 and December 2016 for medications for respiratory and/or allergic diseases and exacerbations (**table I**), from a sample of patients from mainland Portugal, aged 15 years and above (**figure 1**). We analysed the prescriptions delivered to patients on persistent respiratory treatment (n = 8798, **figure 1**) and we considered different prescriptions ordered by the same prescriber, for the same patient, on the same day, as a unique prescription (n = 39810, **figure 1**).

Variables

Persistent respiratory treatment (PRT) was defined as having prescriptions for more than 2 packages of any of the six classes of respiratory maintenance medications: inhaled corticosteroids (ICS) alone or in fixed-dose combination with long-acting beta2 agonists (LABA); leukotriene receptors antagonists (LTRA); long-acting muscarinic antagonist (LAMA) alone or in a fixed-dose combination with LABA or LABA alone.

Table I *- Frequency of prescribed packages of medication for respiratory diseases and exacerbations.*

Medication classes	**Packages n = 312527**	
	n	**%**
Maintenance		
ICS + LABA	37 007	11.8
LTRA	21 085	6.7
LAMA alone	15 897	5.1
LABA alone	10 738	3.4
ICS alone	10 368	3.3
LABA + LAMA	8 051	2.6
Relievers		
SABA alone	8 730	2.8
SAMA alone	5 639	1.8
SABA + SAMA	303	0.1
Exacerbation/infection markers		
Antibiotics	55 810	17.9
OCS	27 399	8.8
Other		
H1-antihistamines (systemic)	73 391	23.5
Expectorant (systemic)	24 857	8.0
Xanthine	8 475	2.7
Cough suppressant (systemic)	4 691	1.5
Cough suppressant with expectorant (systemic)	81	0.0
Anti-Immunoglobulin E	5	0.0

ICS: inhaled corticosteroids; LABA: long-acting beta2 agonists; LTRA: leukotriene receptors antagonists; LAMA: long-acting muscarinic antagonist; SABA: short-acting beta 2 agonist; SAMA: Short-acting muscarinic-antagonist; OCS: oral corticosteroids.

Figure 1 *- Flowchart of patients and prescriptions (adapted from Sá-Sousa et al. (17)).*

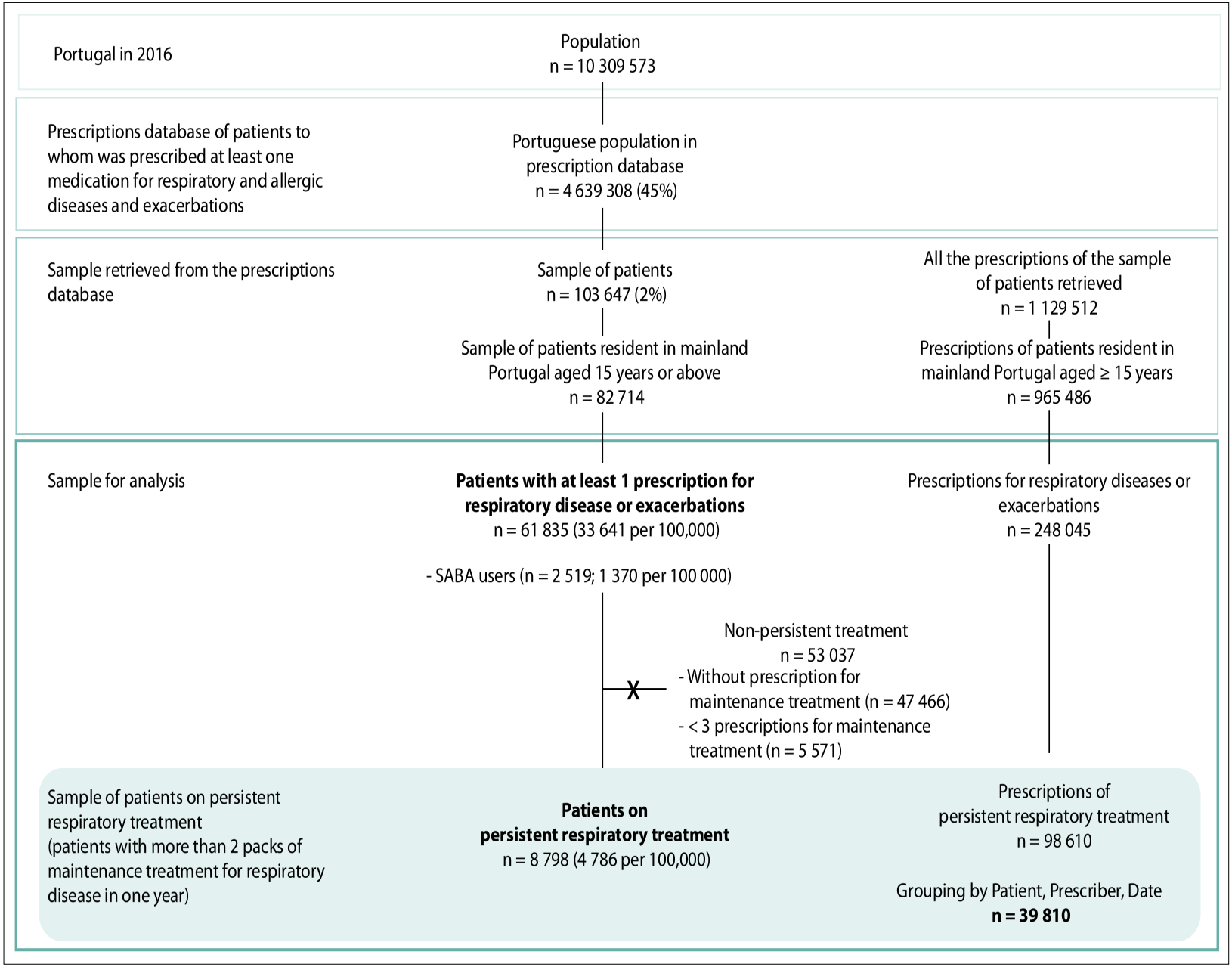


Table II *- Characteristics of the analysed prescriptions (n = 39810).*

	Total Column%, 95%CI	Cluster 1	Cluster 2	Cluster 3	Cluster 4	Cluster 5	Cluster 6	Cluster 7	Cluster 8	Cluster 9	Cluster 10	Cluster 11
		(9,5%)	(6,6%)	(8,3%)	(6,0%)	(13,1%)	(7,6%)	(12,0%)	(5,8%)	(6,7%)	(14,2%)	(10,3%)
Age, med	68	74	63	66	75	68	63	70	44	72	63	66
P25-P75	52-78	64-82	47-76	48-79	65-82	52-78	47-74	58-80	30-59	63-80	50-78	49-77
Region												
South	45.4	8.7	7.7	8.3	6.0	10.2	6.7	12.4	7.6	6.7	14.0	11.6
	44.9-45.9	8.3-9.1	7.4-8.1	7.9-8.7	5.7-6.4	9.8-10.6	6.4.-7.1	11.9-12.9	7.2-8.0	6.4-7.1	13.5-14.5	11.1-12.0
North	32.4	11.0	5.6	9.2	4.1	15.3	7.6	12.6	4.4	6.6	14.2	9.5
	31.9-32.8	10.4-11.5	5.2-6.0	8.7-9.7	3.8-4.5	14.7-15.9	7.1-8.0	12.0-13.1	4.1-4.8	6.2-7.0	13.6-14.8	9.0-10.0
Centre	22.2	8.8	5.5	6.8	8.7	15.9	9.5	10.2	4.1	6.7	14.6	9.1
	21.8-22.6	8.3-9.5	5.1-6.0	6.3-7.4	8.2-9.3	15.2-16.7	8.9-10.1	9.6-10.9	3.7-4.5	6.2-7.2	13.8-15.3	8.5-9.7
Healthcare unit												
Primary care	48.3	11.0	0.3	7.8	5.7	22.7	12.4	14.6	0.0	6.5	19.3	0.1
	47.8-48.8	10.6-11.5	0.3-0.4	7.5-8.2	5.4-6.1	22.1-23.3	11.9-12.9	14.1-15.1	0.0-0.0	6.2-6.9	18.7-19.9	0.0-0.2
Secondary care	21.7	8.3	14.1	10.7	6.9	0.9	0.3	10.1	10.0	8.9	6.0	23.8
	21.3-22.1	7.7-8.9	13.4-14.9	10.1-11.4	6.4-7.5	0.7-1.1	0.2-0.5	9.4-10.7	9.4-10.6	8.3-9.5	5.5-6.6	22.9-24.7
Other	30.0	7.9	11.1	7.2	5.9	6.8	5.3	9.3	12.0	5.3	12.1	17.1
	39.6-30.4	7.4-8.4	10.5-11.7	6.8-7.7	5.5-6.3	6.4-7.3	4.9-5.7	8.8-9.8	11.4-12.6	4.9-5.7	11.5-12.7	16.4-17.7
Healthcare provider												
Public	69.7	10.1	4.6	8.7	6.1	15.8	8.6	13.1	3.1	7.3	15.1	7.5
	69.2-70.1	9.8-10.5	4.4-4.9	8.4-9.1	5.8-6.3	15.4-16.2	8.2-8.9	12.7-13.5	2.9-3.3	7.0-7.6	15.7-15.5	7.2-7.8
Private	30.3	7.9	11.0	7.2	5.9	7.0	5.4	9.4	11.9	5.3	12.2	16.9
	29.8-30.8	7.4-8.4	10.4-11.5	6.7-7.6	5.5-6.4	6.5-7.4	5.0-5.8	.9-10.0	11.3-12.5	4.9-5.7	11.6-12.8	16.2-17.6

Frequencies are summarized as row % and 95% Confidence Interval (95%CI), otherwise is indicated; P25-P75: Percentiles 25-75.

1. *Medication type* – active substances were classified in 14 medication types according to the International Non-proprietary Names: ICS plus LABA (ICS + LABA); LTRA; ICS alone; LABA alone; SABA alone; LAMA alone; LABA plus LAMA (LABA + LAMA); Xanthine; (short-acting muscarinic antagonists (SAMA) alone; SABA plus SAMA (SABA + SAMA). For a better understanding of the clinical sense of the clusters, we additionally included Antibiotics; OCS; H1-antihistamine (Anti-H1); nasal corticosteroids (nCS) and Expectorants combined or not with Cough suppressants in the analysis.
2. *Prescribers' specialties* – the specialties (n = 52) were grouped in general practitioners (GPs), pulmonologists, allergists, internists, and the other, less frequent, specialities grouped as "other".
3. *Packages* – number of packages of each medication type prescribed. In the BDNP system, it is possible to include several packages for each medication in the same prescription.

Additional external variables were analysed, such as the age of the patient; region of the prescription (mainland Portugal has 5 NUTS II regions that were recorded in 3: North, Center and South (Lisbon, Algarve and Alentejo)); Healthcare unit (primary care, secondary care or other) and healthcare provider (public or private).

Cluster analysis

Cluster analysis techniques were applied to identify prescription patterns based on medication and specialty of the prescriber using a two-step approach. The variables included in the final model were medication type (ICS + LABA; LTRA; ICS alone; LABA alone; SABA alone; LAMA alone; LABA + LAMA; Xanthine; SAMA alone; SABA + SAMA); and the specialty of the prescribers (GPs; pulmonologists; allergists; internist; other). In the first step, an automatic clustering algorithm estimated the number of clusters that best fitted the data, based on the Bayesian Information Criterion. This estimate was then used for the clustering analysis based on log-likelihood distance measures (18). We selected the parameters for which the model had the highest quality, and the final model had a silhouette coefficient of 0.5. The presence of additional medication (Antibiotics, OCS, anti-H1, nCS and expectorants combined or not with cough suppressants) was explored for each cluster.

Statistical analysis

Categorical variables are presented as absolute frequencies and proportions and 95% Confidence Interval for proportion (95% CI). Age differences between clusters were tested by Kruskal-Wallis chi-square. Statistical significance was set for a P-value of less than 0.05.

IBM SPSS Statistics 25 was used to conduct the two-step cluster analysis and RStudio 1.1.456 (https://rstudio.com/) for pre-processing and other analyses.

Results

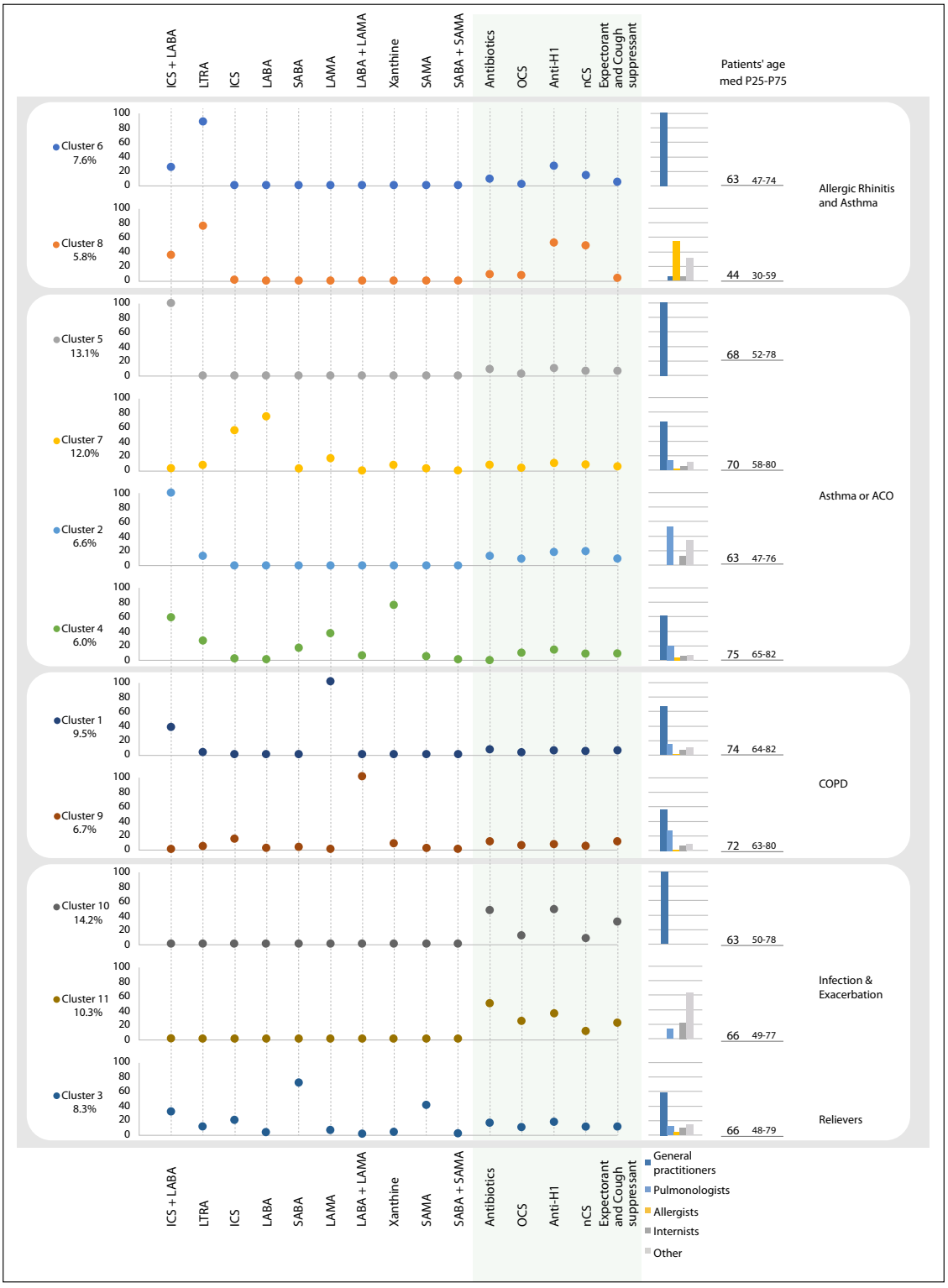
A total of 39810 prescriptions of PRT (**figure 1**) were registered in 2016 for the analysed sample, corresponding to 312527 packages (**table I**). Maintenance treatment represents 1/3 of the prescribed packages, mostly for ICS + LABA (11.8%) and LTRA (6.7%). Globally, the most prescribed drugs were H1-antihistamines (23.5%) and antibiotics (17.9%).

The cluster analysis conducted to assess prescription patterns based on medication and specialty of the prescriber, revealed that an eleven-cluster model was the solution that best fitted our data. The characteristics of prescriptions and external variables are described in **table II**. The most frequent prescription patterns are grouped in clusters 10 and 5, prescribed exclusively by GPs, and in clusters 7 and 11, written by prescribers with different specialties. The clusters' characteristics are summarized in **figure 2** and **online supplements table IS**. Additional medication (Antibiotics, OCS, anti-H1, nCS and expectorants combined or not with cough suppressants) and patients' age are also presented for each cluster.

Regarding external variables (**table II**), Cluster 8 was the pattern prescribed to youngest patients ($p < 0.001$) and clusters 1 and 4 to the oldest ($p < 0.001$). At primary care units and public healthcare providers, the most frequent prescriptions are grouped in Cluster 5 or 10, whereas secondary healthcare services and private providers prescriptions are grouped in cluster 11 more often. Based on the clinical interpretation of the medication in each cluster, including patients' age, they were grouped into four subsets, as follows:

1) *Medication for possibly Allergic Rhinitis and Asthma*:
   Clusters 6: prescriptions for LTRA alone or combined mostly with ICS + LABA. Additional frequent medications were H1-antihistamine (anti-H1) and nCS. Prescribed GPs for patients with a median age of 63 years old.
   Cluster 8: prescriptions for LTRA alone or combined mostly with ICS + LABA. Additional frequent medications were anti-H1 and nCS. Prescribed by allergists for patients with a median age 44 years old.
2) *Medication for possibly Asthma or ACO*:
   Clusters 5: prescriptions for ICS + LABA fixed combination, prescribed exclusively by GPs for patients with a median age of 68 years old.
   Cluster 7: prescriptions for ICS, LABA and LAMA. Prescribed mostly by GPs for patients with a median age of 70 years old.
   Cluster 2: prescriptions for ICS + LABA alone or combined with LTRA, and additionally includes prescriptions for anti-H1 and nCS. Prescribed mostly by pulmonologists for patients with a median age of 63 years old.
   Cluster 4: prescriptions for ICS + LABA, Xanthines, LAMA and LTRA. Prescribed mostly by GPs for patients with a median age of 75 years old.

Figure 2 *- Frequency of each prescription cluster (%) determined by 2 step cluster analysis and distribution of medication types, prescribers' specialities and age of the patients in each cluster. The distribution of additional medication, not included in the model, is presented in shadow.*



3) *Medication for possibly COPD*:
   Cluster 1: prescriptions for LAMA alone or combined with ICS + LABA. Prescribed mostly by GPs for patients with a median age of 74 years old.
   Cluster 9: prescriptions for LABA + LAMA alone or combined with ICS. Prescribed mostly by GPs and pulmonologists for patients with a median age of 72 years old.
4) *Medication for infection, exacerbation and relievers of symptoms*:
   Cluster 10: prescriptions for antibiotics, OCS, anti-H1, nCS and expectorants with cough suppressants, with no maintenance treatment. Prescribed exclusively by GPs for patients with a median age of 63 years old.
   Cluster 11: prescriptions for antibiotics, OCS, anti-H1, nCS and expectorants with cough suppressants, with no maintenance treatment. Prescribed mostly by specialties not related to respiratory diseases for patients with a median age of 66 years old.
   Cluster 3: prescription mainly for SABA, SAMA, but also with ICS + LABA, ICS, LTRA and LAMA. Prescribed mostly by GPs for patients with a median age of 66 years old.

Discussion

Eleven different prescriptions patterns clusters were revealed by unsupervised analysis based on medications and prescribers' specialties, and these clusters were grouped in four, based on the theoretical therapeutic indications of the medications and patient's age in each cluster.

Comparing the clusters obtained by unsupervised analyses with the pharmacotherapy recommended in relevant guidelines for asthma (1), COPD (2), and allergic rhinitis and asthma (19), we found that they have clinical relevance. According to Global Initiative for Asthma (GINA), in a stepwise approach, if the response to the treatment is suboptimal, it is recommended to intensify the treatment, either by increasing the dose of currently used ICS and adding another controller medication, such as LABA, LTRA, and xanthines. On the other hand, Allergic Rhinitis and its Impact on Asthma (ARIA) (19) recommends the treatment with nCS with either anti-H1 or LTRA for seasonal allergic rhinitis. Cluster 6 and 8, are profiles that closely resemble the GINA and ARIA recommendations for allergic asthma and rhinitis.

Guidelines advise different COPD initial treatments depending on the severity of symptoms, exacerbations, and airflow limitation (2). It consists of a bronchodilator, either SABA or SAMA or LABA or LAMA and LABA or LAMA; and, if the symptoms persist, both LABA + LAMA or ICS + LABA. For more severe cases the recommended initial therapy is LAMA + LAMA or, in patients with a history suggestive of asthma-COPD overlap or based on eosinophilic counts, ICS + LABA. The higher level of pharmacological care corresponds to triple therapy with LAMA + LABA + ICS or add-on of phosphodiesterase-4 inhibitor or a macrolide. Clusters 1 and 9 are profiles matching GOLD recommendations for COPD management.

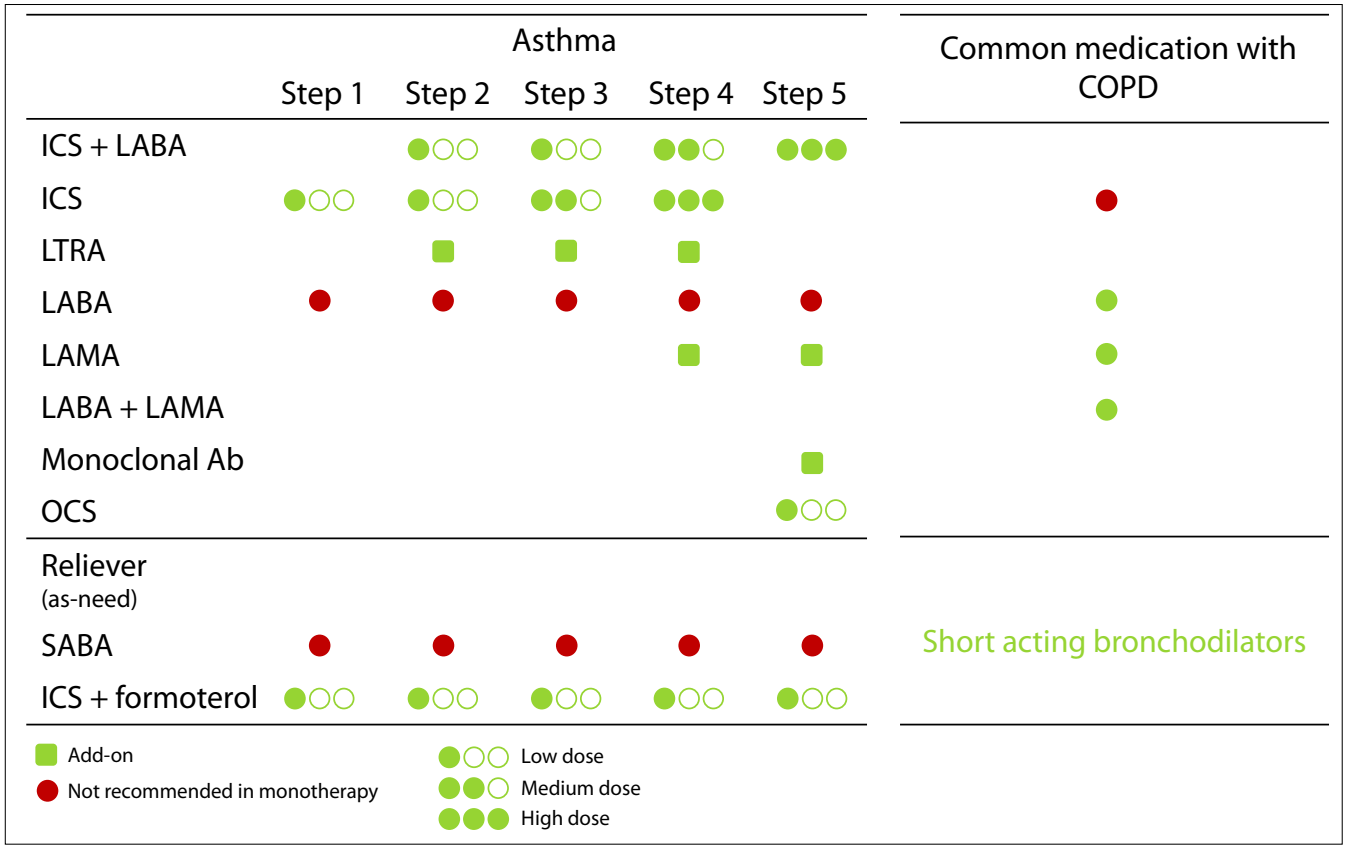
COPD therapeutic options have similarities with asthma treatment (**figure 3**). The higher level of asthma care corresponds to treatment with a high dose of ICS + LABA and the add-on LAMA, Immunoglobulin E (IgE), a low dose of OCS or biological therapy (1). The GINA recommendations for treating patients with features of both asthma and COPD is ICS in a low or moderate dose and add-on treatment with LABA and/or LAMA. Clusters 2,4,5 and 7 are mixed profiles corresponding to medication for possible asthma or ACO.

Until 2019, GINA recommended the use of SABA as the first line of asthma treatment (20). The recently published guide for asthma management by the GINA network, recommends that ICS should be used whenever SABA is used, and ICS combined with formoterol may be used in low dose as a reliever option (**figure 3**) (1). Cluster 3 describes a profile corresponding to rescue medication for asthma and COPD; clusters 10 (exclusively prescribed by GPs) and 11 (mostly prescribed by specialties not related to respiratory diseases) are profiles for exacerbations and infection treatment. This indicates that in some clinical visits, patients on PRT only receive a prescription for infections and exacerbations and that the use of some of these medications may be related to other comorbidities.

Studies that use prescription claims as proxies for diagnosis of asthma and COPD, based on *a priori* established algorithms, are controversial. Weidinger *et al.* used a representative sample of patients registered in primary healthcare units in Sweden to show that there was a large discrepancy between the proportion of patients with medication for asthma and COPD (SABA, LABA, ICS, and fixed combinations of ICS + LABA) with the proportion of patients with a formal diagnosis for asthma or COPD (5). These results indicate that the use of prescriptions as a proxy for the diagnosis may not be accurate. However, another study on Dutch children diagnosed with atopic diseases reported that having two or more prescriptions for asthma, including ICS can be a reliable proxy for asthma (6). A systematic review of studies on the classification of asthma severity using claims data stated that no best theory-driven algorithm has been established so far (7).

On the other hand, unsupervised methods, not based on *a priori* assumptions, bring new insight into the identification of patterns clinically relevant and with several applications. Slobbe *et al.* have shown that unsupervised methods applied to medication claims, may be used to predict the prevalence of six diseases, including asthma and COPD (21). Another study used clustering methods to establish different profiles of patients based on airflow limitation and explore its characteristics, namely in terms of medication prescribed in each cluster of adult patients with mild-to-moderate airflow limitation from the Korean National Health and Nutrition Examination Survey (16). Clustering methods have also been used to explore adherence barriers among respiratory patients, towards personalized care. A study using clusters based on adherence to inhalers in COPD patients, shown that certain de-

Figure 3 *- Medication used in asthma management and common medication with COPD.*



mographic and clinical measurements, including lung function, cough and cognitive impairment, were determinants for different profiles of adherence (22). To the best of our knowledge, there are no studies using unsupervised methods with similar methodology and variable to support our results.

This was the first analysis of the patterns of respiratory medication in the official Portuguese prescription database. Nevertheless, the present study has several limitations. The main limitation is related to the lack of information regarding treatment indication and duration of the treatment. Although we obtained prescription patterns with clinical relevance for asthma and COPD identification, having the diagnosis would allow the validation of the clustering method. Moreover, adding the indication could raise evidence on the medications commonly used for different indications and also used as off-label in the real-world. The duration of the treatment is also important for patient profiling, especially for exacerbation markers such as antibiotics and OCS. As with any data-driven clustering, there are limitations in the interpretation of derived classes as being a true set of clinically meaningful subgroups (9). Finally, despite the large size of the analysed sample, it may not be representative of the Portuguese patients' population, because we were not able to analyse the complete dataset of the BDNP.

The clusters encountered in this study may be useful to explore primary adherence differences between patterns of prescriptions and also to compare with OTC (Over-the-counter) patterns. To address the goals of management of chronic respiratory diseases, besides giving the appropriate prescription for each condition, factors such as adherence to the treatment and use of over-the-counter medication need to be optimized. RWD has contributed to a better understanding of primary nonadherence (23, 24) and to raise awareness on the use of OTC medication for relievers of asthma symptoms (25). However, OTC uses of medication are not registered on the BDNP database and to the best of our knowledge, there is no data available on OTC medication for respiratory diseases in Portugal. In the future, studies on primary adherence, and also on OTC medication may uncover important barriers to adequate management of disease in the Portuguese population.

Conclusions

This study was based on prescription claims and revealed 11 prescription patterns for respiratory medication. These patterns could be grouped into four profiles medication for possibly: 1) Allergic Rhinitis and Asthma, 2) Asthma or ACO, 3) COPD, and 4) infection, exacerbation and relievers of symptoms medication and according to the prescribers' specialties. This profiling is the first step to understand the Portuguese reality on the prescribing of respiratory medication.

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Conflict of interests

The authors declare that they have no conflict of interests.

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Table IS *- Distribution of medication types and prescriber specialities by prescription clusters, determined by 2 step cluster analysis.*

Clusters	1	2	3	4	5	6	7	8	9	10	11
Medication type, %											
ICS + LABA	37.1	100.0	32.0	57.8	100.0	25.3	1.9	36.4	0.0	0.0	0.0
LTRA	2.8	11.7	11.2	26.8	0.0	100.0	7.5	75.1	4.7	0.0	0.0
ICS	0.0	0.0	20.3	1.3	0.0	0.0	54.8	0.9	14.9	0.0	0.0
LABA	0.0	0.0	3.7	1.0	0.0	0.0	74.2	0.0	2.0	0.0	0.0
SABA	0.0	0.0	71.2	15.9	0.0	0.0	2.9	0.0	3.4	0.0	0.0
LAMA	100.0	0.0	5.2	36.7	0.0	0.0	16.7	0.0	0.9	0.0	0.0
LABA + LAMA	0.0	0.0	1.1	6.1	0.0	0.0	0.0	0.0	100.0	0.0	0.0
Xanthine	0.0	0.0	2.7	75.7	0.0	0.0	6.8	0.0	8.7	0.0	0.0
SAMA	0.0	0.0	40.6	4.1	0.0	1.4	1.7	0.1	1.4	0.0	0.0
SABA + SAMA	0.0	0.0	1.9	0.5	0.0	0.0	0.1	0.0	0.2	0.0	0.5
Antibiotics	6.4	12.1	16.7	0.0	8.1	8.3	6.8	9.3	10.8	46.3	49.4
OCS	3.7	8.4	10.1	9.4	2.3	2.0	3.4	7.7	5.2	12.1	25.1
AntiH1	5.8	17.8	17.0	13.0	9.8	26.0	10.2	52.3	6.3	48.3	34.9
nCS	4.2	19.3	10.6	8.1	6.4	13.0	7.7	49.1	4.2	8.2	10.5
Expectorant and Cough suppressant	6.1	9.0	10.3	8.0	5.7	5.0	5.0	4.0	10.5	31.5	21.6
Prescriber specialty, %											
General practitioners	66.3	0.0	58.5	60.5	100.0	100.0	67.3	0.0	56.4	100.0	0.0
Pulmonologists	15.3	52.7	11.9	20.1	0.0	0.0	13.2	6.3	27.4	0.0	13.2
Allergists	1.0	0.0	4.1	4.6	0.0	0.0	1.8	55.2	0.3	0.0	0.0
Internist	7.0	13.0	10.3	6.4	0.0	0.0	5.9	6.2	6.7	0.0	23.0
Other	10.4	34.3	15.3	8.4	0.0	0.0	11.8	32.3	9.1	0.0	63.8

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Failure of desensitization with Pfizer-BioNTech COVID-19 vaccine in two asthmatic patients

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Key words

Desensitization; vaccine; COVID19; asthma; adverse drug reaction.

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Summary

Since December 2020, in various countries of the world, many cases of severe allergic reactions after administration of PfizerBioNTech COVID-19 vaccine, were reported. A great concern has arisen among the doctors who administer the vaccine and the allergic patients who undergo vaccinations. In Italy guidelines were published in order to stratify the risk in the allergic population.

In mRNA vaccines, the component currently suspected of causing allergic reactions is the polyethylene glycol excipient (PEG or macrogol). In patients who have shown an immediate allergic reaction to vaccine and who are negative to skin tests for PEG, desensitization with the same vaccine is proposed. In this paper we describe two cases of asthma after the first COVID vaccine administration in which desensitization has failed.

Impact statement

Subjects with particular conditions, such as asthma, can carry out the anti COVID vaccination, but need a more specific and individualized management.

Introduction

As of December 23, 2020, 175 case reports were identified as possible cases of severe allergic reactions in the United States, including anaphylaxis, after administration of PfizerBioNTech COVID-19 vaccine (1). The median interval from vaccine receipt to symptom onset was 13 minutes (range 2-150 minutes). Among persons with follow-up information available, all had recovered or been discharged home. Most of the patients had suffered from a prior history of allergy or anaphylaxis.

Subsequently, millions more doses of Pfizer-BioNTech vaccine were administered, with an updated reported anaphylaxis rate of 4.7 cases per 1 million doses (2).

Furthermore, a recent study has found the vast majority of people who have a prior history of anaphylaxis are unlikely to have a serious adverse reaction after receiving Pfizer's COVID-19 vaccine (3). In the updated AIFA (Italian Drug Agency) report on the surveillance of COVID-19 vaccines, out of 84.010.605 doses of vaccine administered in Italy in the period between 12/27/2020 and 09/26/2021, were reported 3 cases of anaphylaxis per million doses of Comirnaty (4).

Nevertheless, a great concern has arisen among the doctors who administer the vaccine and the allergic patients who undergo vaccinations. In Italy, guidelines were readily published in order to stratify the risk in the allergic population. Specific guidelines are given on the management of allergic reactions to the vaccine (5) and rule out allergy to polyethylene glycol (PEG), present in Pfizer vaccines to help stabilize the mRNA, a possible cause of these reactions. In patients who have shown an immediate allergic reaction and who are negative to skin tests for PEG and polysorbates (6), compounds structurally related to PEG, desensitization with the same vaccine is proposed, according to the guidelines proposed by EAACI (7). We describe below two cases of asthma after the first COVID vaccine administration in which desensitization has failed.

Case 1

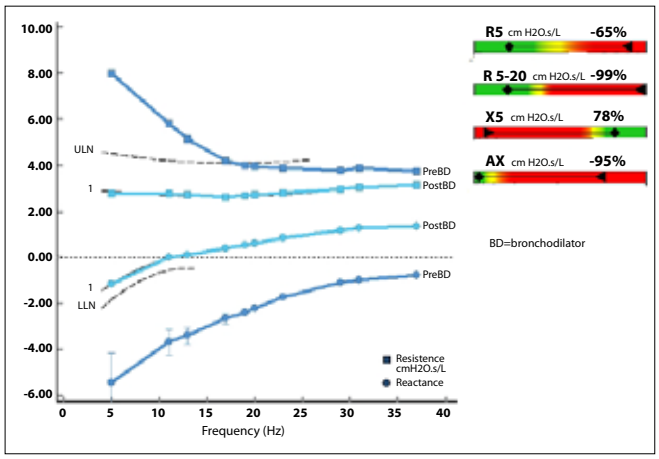
ML is a 60-year-old, nonsmoker woman, suffering from asthma in PET allergy (not present at home). In March 2020 after SARS CoV2 infection she developed an exacerbation of asthma treated with oral steroids. After this episode she started LABA/ICS therapy. In January 2021 she had immediate mild urticaria and asth-

ma (dyspnea and cough) after the first dose of Pfizer-BioNTech vaccine. The patient underwent skin tests to rule out allergy to the vaccine additives, with negative results. Subsequently she underwent desensitization. Asthma was in good control with LABA/ICS therapy, and Asthma Control Test (ACT) and oscillometry were normal. We performed desensitization starting with 0.05 mL of the 1:10 dilution of the vaccine and then with 0.05 ml – 0.1 ml – 0.15 ml of the undiluted vaccine every 20 minutes. She developed asthma at the last dose of the vaccine. The patient was subjected to oscillometry, which showed an increase in peripheral and central resistances and reactance, then she was treated with intravenous steroids and inhaled ICS/formoterol, with significant reversibility after administration of therapy (**figure 1**).

Case 2

FL is a 44-year-old professional nurse with atopic asthma to dust mites and grasses well controlled with daily low inhaled corticosteroid according to the 2020 Global Initiative for Asthma (GINA) guidelines approved at the time of the desensititazion. She has no known diagnosis of COVID-19 disease. In January 2021, a few minutes after the Pfizer-Biontech COVID-19 vaccine, she experienced a respiratory reaction (dry cough and a sensation of a lump in the throat) with biphasic trend. The PEG and polysorbate skin testing were negative. We carried out desensitization with Pfizer-BioNTech vaccine previously performing an asthma control test that indicated a well- controlled asthma, because lung function test was not available due to the COVID-19 pandemic. During the subsequent immunization in graded doses, she showed cough and tightness in the throat twenty minutes after the third dose (0.1 mL of the indiluted vaccine). The symptoms resolved after treatment with intravenous steroid and antihistamine. Desensitization was discontinued.

Figure 1 *- Oscillometry modification in case 1: the increase in peripheral and central resistances and reactance, and the significant reversibility after administration of therapy.*



Conclusions

No current research highlights that the COVID-19 vaccines worsen asthma symptoms. Not even all immediate reactions that occur in association with vaccines are true allergic reactions. This is described in a CDC report demonstrating that out of 175 possible severe allergic reactions, 86 (49%) were non anaphylactic allergic reactions (1). People with asthma, as with other vaccine recipients, may experience temporary side effects after getting the vaccine, like fever or flu-like symptoms, which can act as an asthma trigger. This could explain the failure of desensitization in the cases described above. We emphasize the importance of asthma stabilization in asthmatic patients who are subject to vaccination, particularly in those with severe asthma and who have a previous history of allergy. Patients who have developed asthma after the first dose of the vaccine should be monitored carefully when the second dose is given, despite negative skin tests for vaccine additives. Another chance is to consider administering an alternative vaccine. At the moment the limitation of diagnostics is due to the impossibility of carrying out tests with the vaccine in its entirety.

Conflict of interests

The authors declare that they have no conflict of interests.

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Patients with elevated basal tryptase serum levels should be tested for hereditary alpha-tryptasemia

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Key words

Tryptase; anaphylaxis; mastocytosis; hereditary alpha tryptasemia; allergy.

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To the Editor,

elevated total basal tryptase levels are not rare in patients presenting to allergy outpatient clinics and often lead to multiple investigations including bone marrow biopsy to exclude myeloid neoplasia and clonal mast cell diseases such as systemic mastocytosis (SM).

Hereditary alpha-tryptasemia (HAT) is a recently described inherited condition associated with elevated basal tryptase levels (1) and characterized by extra copies of the alpha tryptase encoding gene *TPSAB1*. Patients may either be asymptomatic or develop a syndrome involving multiple organ systems and characterized by symptoms similar to those of mast cell activation syndrome or SM (2). The diagnosis of HAT can be challenging and requires a careful analysis of the *TPSAB1* and *TPSB2* copy number variation (CNV). The total copy number of *TPSAB1* and *TPSB2* for normal individuals is four; individuals with a duplication in the *TPSAB1* gene have a total copy number of five or more (3).

Here we present four cases with sustained elevated basal tryptase levels without obvious explanation in whom digital droplet polymerase chain reaction (ddPCR) revealed HAT. All patients signed an informed consent.

Case 1: a 25-year-old patient presented with an anaphylactic reaction grade 3 (according to UR Mueller (4)) following seafood ingestion. Skin testing and IgE analysis did not detect any sensitization to fish, seafood or anisakis. Total tryptase levels were elevated (17 μg/l, normal value < 11.4 μg/l) initially and on several occasions in the following years, during which the patient was in perfect health. In 2020, *TPSAB1* CNV analysis by ddPCR showed a calculated alpha-tryptase copy number of 2 and calculated beta-tryptase copy number of 3, consistent with extra allelic alpha-tryptase copies of *TPSAB1* and the diagnosis of HAT. The patient refused a bone marrow biopsy. However, based on the benign clinical course, a diagnosis of advanced SM was very unlikely.

Case 2: a 43-year-old patient presented for a grade 3 anaphylactic reaction of unknown cause. Total tryptase level was 35 µg/l. Two months later, allergic workup including skin testing and IgE analysis was inconclusive, the tryptase level was still elevated (20.5 µg/l). He complained of persistent abdominal pain, pruritic wheals and asthenia. He underwent a full work-up for suspected SM including bone marrow biopsy, next-generation sequencing for a panel of 54 genes known to be linked to hematological neoplastic disease, peripheral blood flow cytometry and c-Kit D816V mutation analysis. A panel of serologies and stood evaluation for parasites, fecal calprotectine, and gastro-duodenoscopy with biopsies were also performed (**table I**). All these investigations were unremarkable except for microcytic anemia, osteopenia and a c.1934delG, pGly645fs mutation in the *ASXL1* gene. The symptoms responded only partially to antihistaminics and cromoglicic acid, a better relief was achieved with omalizumab. A diagnosis of idiopathic mast cell activation syndrome was suspected and a close follow-up because of the *ASXL1* mutation was suggested. During the next two years, no hematological disease developed. In 2020, testing for *TPSAB1* CNV by ddPCR revealed a calculated alpha-tryptase copy number of 4 and the calculated beta-tryptase copy number was 2, consistent with the diagnosis of HAT.

Case 3: 58-year-old HIV infected patient presented with a pruritic maculopapular erythematous rash of unknown origin. He reported having perennial rhinoconjunctivitis and throat pruritus following the ingestion of nuts. Allergic workup showed sensitization to tree pollen, grass pollen and house dust mites. Dermatological investigations ruled out cutaneous mastocytosis and were consistent with parapsoriasis. TCR rearrangement analysis did not show a clone supporting the presence of mycosis fungoides. Patch tests were negative for all suspected allergens. Phototherapy was introduced with good response. Tryptase was assessed and found increased at 17.7 µg/l. Levels were stable during four subsequent controls over the following year. *TPSAB1* CNV analysis by ddPCR showed a calculated alpha-tryptase copy number of 2 and a calculated beta-tryptase copy number of 3, consistent with HAT.

Table I *- Patient's characteristics and digital droplet PCR results.*

	Clinical characteristics	**Biological and radiological findings**	**ddPCR result**
Patient 1. M, 25 yo.	Grade 3 anaphylaxis after fish consumption. No signs of cutaneous mastocytosis. Comorbidities: - Atopic dermatitis with type I sensitisation to cat and dog dander. - Ulnar compressive neuropathy.	Unremarkable peripheral blood counts and flow cytometry. No D816V c-KIT mutation in the peripheral blood. No paraprotein, negative ANA titer. Tryptase values 2018-2020: 17.1, 18.7, 16.9, 19.4 µg/l.	Calculated alpha-tryptase copy number is 2; calculated beta-tryptase copy number is 3. Total of 5 copies.
Patient 2. M, 43 yo.	Grade 3 anaphylactic reaction of unknown cause. Persistent abdominal pain, arthralgia, pruritic wheals and asthenia since the anaphylactic reaction. No signs of cutaneous mastocytosis. Comorbidities: - Depression. - No atopy. - Beta thalassemia minor.	Unremarkable thoraco-abdominal CT scan, gastroduodenal endoscopy, peripheral blood counts, bone marrow biopsy and flow cytometry. No signs of parasitic infection. Osteopenia in bone densitometry. *ASXL1* gene mutation. No D816V c-KIT mutation in the peripheral blood. Tryptase values 2017-2021: 35.0, 20.5, 20.0, 23.5, 22.3, 15.5, 24.8, 16.6, 30.2, 23.6, 27.2, 26.2, 30.9 µg/l.	Calculated alpha-tryptase copy number is 4; calculated beta-tryptase copy number is 2. Total of 6 copies.
Patient 3. M, 58 yo.	Maculopapular erythematous skin lesions and pruritus of unknown origin. No signs of cutaneous mastocytosis. Comorbidities: - Allergic rhinoconjunctivitis and nut allergy. - Parapsoriasis. - HIV infection.	Normal total IgE levels, negative ANCA, normal peripheral blood cell counts, no paraprotein, no D816V c-KIT mutation in the peripheral blood. HIV1 5.6E1 copies/ml. $CD4^+$ 482/µl. Tryptase values 2019-2020: 17.7, 17.5, 17.2, 18.8 µg/l.	Calculated alpha-tryptase copy number is 2; calculated beta-tryptase copy number is 3. Total of 5 copies.
Patient 4. F, 52 yo.	Skin rash after administration of contrast media for a CT scan. No signs of cutaneous mastocytosis. Comorbidities: - Amoxicillin-clavulanic acid allergy	Blood counts and chemistries unremarkable. No D816V c-KIT mutation in the peripheral blood. Tryptase values 2019-2020: 14.1, 15.7, 15.9 µg/l.	Calculated alpha-tryptase copy number is 3; calculated beta-tryptase copy number is 2. Total of 5 copies.

Case 4: a 53-year-old woman presented with a skin rash that occurred after administration of contrast media for a CT scan and antibiotics given for pneumonia. Skin tests with these compounds were negative. However, lymphocyte transformation test was positive for amoxicillin-clavulanic acid. Tryptase was increased in several occasions during the next year. Analysis of *TPSAB1* CNV by ddPCR revealed a calculated alpha-tryptase copy number of 3 and a calculated beta-tryptase copy number was 2, consistent with HAT.

The four cases described here (**table I**), with no familiar history of mast cell-related disease, illustrate that HAT might be more common than previously considered, as recently reported in an unselected British birth cohort where 5% had a raised *TPSAB1* copy number (3, 5). Thus, *TPSAB1* CNV should be tested in cases with elevated basal tryptase levels without obvious explanation such as end-stage kidney disease, helminth infections, and myelodysplastic/myeloproliferative disease (6). As to mastocytosis, SM in particular, a high prevalence of increased *TPSAB1* copy numbers has been reported (> 15%), indicating a potential pathogenic role of HAT and an elevated risk of severe anaphylaxis (7, 8). This underscores the utility of testing for *TPSAB1* CNV in SM or in cases of severe anaphylaxis, as illustrated in our cases 1 and 2. Nevertheless, which patient qualifies for *TPSAB1* CNV testing is currently a matter of debate. HAT has been described in patients with basal tryptase levels < 11.4 μg/l (2), but not < 7.6 μg/l (3). On the other hand, we observed two patients exhibiting elevated basal tryptase levels of unknown origin and allergic reactions with normal *TPSAB1* copy numbers. Thus, the impact of HAT on the clinical management needs further elucidation, as increased copy numbers of *TPSAB1* seem to have a variable clinical penetrance and definitely do not rule out concomitant SM. Thus, awaiting results from larger studies on the long-term prognosis of HAT, *TPSAB1* CNV testing should not replace, but rather be added to the diagnostic work-up of elevated tryptase levels. Regarding the treatment for HAT, it becomes progressively clear that most patients will stay asymptomatic while only a minority, as case 2, will require more intensive therapy, *e.g.*, with omalizumab, for mast cell stabilization (9).

In conclusion, the routine availability of a genetic test for HAT will help to identify a particular population of patients among those with elevated basal tryptase levels of hitherto unknown cause. The clinical significance of HAT, in particular whether these patients require a close follow-up and specific treatment due to an increased risk for severe anaphylaxis or SM, remains to be studied.

Conflict of interests

The authors declare that they have no conflict of interests.

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