Laboratory data

The median (IQR) *Apis mellifera* sIgE levels of our cohort were 6.7 (1.0-20.3) kU/L. In our sub-group of VIT patients, pre-treatment levels were higher (13.7; 5.3-43.5), dropping significantly during follow-up to 3.3 (1.1-7.9) kU/L.

Regarding molecular sIgE sensitization, patients were tested for *Api m 1* (n=73)*, Api m 4* (n=42) and *Api m 10* (n=64). Overall, *Api m 1* was positive in 42 patients (57.5%) and *Api m 10* in 32 (50.0%), whereas only 5 patients (11.9%) had positive *Api m 4* levels. Three (5.3%) patients of all cohort were sensitized to all three allergens. Median (IQR) sIgE levels for *Api m 1, 4* and *10* were 0.47 *(*0.10-1.57*),* 0.01 *(*0.00-0.08*)* and 0.32 (0.08-1.62), respectively.

In the VIT subgroup, among patients whose molecular components were available, 18 (72.0% of 25 measurements) were sensitized to *Api m 1*, and 15 (65.2% of 23) to *Api m 10*, in contrast with only 2 (10.5% of 19) patients with positive sIgE to *Api m 4.* These same two patients were also polysensitized to the other molecular allergens. Detailed sensitizations to molecular allergens are depicted in **Tables I.**

Associations between systemic reactions and demographic, clinical or laboratory variables

1. *Severity of index-reaction to bee sting was associated with age and molecular sensitization profiles to Apis mellifera*

Severity of the reaction at allergy onset was classified in 85 patients. There was a weak but significant positive correlation with age at onset, with older patients presenting more severe reactions (Spearman's coefficient [*rho*]=0.249*, p*=0.040). Inversely, patients that were only sensitized to *Api m 1* (among the three

measured proteins) had milder reactions in comparison to non-monosensitized patients – 2 (*IQR* 2-2*)* vs 3 (*IQR* 3-4), p=0.015. When fitted in a multivariate linear regression model that included variables with *p*<0.100 (age, atopic comorbidities and sensitization solely to *Api m 1*) and used a backward stepwise approach, the *Api m 1* mono-sensitization profile retained its significance (*p*=0.031) and was considered an independent predictor for milder systemic index-reactions to bee sting. Statistical analysis for all demographic, clinical and laboratory variables is described in **Table II.**

2. *Occurrence of systemic reactions during VIT was associated with sensitization to Api m 10*

Patients that concluded or were still undergoing VIT were analyzed. No significant associations were found between demography, clinical and laboratory variables and the proportion of VIT patients with systemic reactions, with one notable exception – patients sensitized to *Api m 10*, regardless of potential co-sensitizations, were significantly more associated with systemic adverse reactions during VIT when compared to non-systemic reactions (90.0 *vs* 46.2%, *p=0.038*). Statistical analysis for potential associations is depicted in **Table III.**

3. *Absence of systemic reactions with bee re-stings was potentially associated with sensitization to Api m 10 in VIT patients*

In the VIT subgroup, no statistically significant associations were found between systemic reactions to re-stings and molecular sensitization profiles. However, re-stung patients non-sensitized to *Api m 10*, regardless of potential co-sensitizations, had a tendency for association with systemic adverse reactions

(25.0 vs 85.7%, *p*=0.093). Additionally, lower levels of *Api m 10* also appeared to be marginally associated with systemic reactions in re-stings (0.15 vs 1.08 kU/l, *p*=0.059). Inversely, *Api m 4* sensitization (in addition to the other two molecular allergens) was marginally associated with systemic reactions (66.7 vs 0.00%, *p*=0.087), but there was no association with sIgE levels. Statistical analysis for potential associations is summarized in **Table IV.**

DISCUSSION

Our study aimed to characterize the clinical and laboratory profiles of a Portuguese cohort of BVA patients. We also sought to establish a relationship between molecular allergic profiles with index reactions' severity, VIT efficacy, and adverse events such as reactions during VIT and re-stings.

Several similarities were shared between our cohort and previously published studies, but there are also notable differences.

Regarding demographic and clinical background, most of our patients were young male beekeepers, which is known to fit with the national profile and is also the occupational activity most commonly associated with BVA (15). Atopic comorbidities were highly prevalent, which has also been observed in other cohorts (16). No patients were diagnosed with mast-cell diseases nor had elevated basal tryptase levels, which are known predisposing factors for anaphylaxis to hymenoptera, but were absent in our cohort (17).

As for molecular allergen profiling, it should be noted that at least half our patients were sensitized to *Api m 1* and/or *Api m 10*, highlighting their importance as major honeybee venom allergens (18–20). Sensitization to *Api m 4*, on the other hand,

was far less common in our cohort (11.9% of measurements). Despite *Api m 4* being mainly defined as a minor allergen, recent studies have reported a higher prevalence of this allergen in comparison with our results (12,13,21). Our acquisition of *Api m 4* for ImmunoCAP measurement has been very recent and, therefore, much of this data was obtained significantly after index-reaction, which may have influenced results. Additionally, some studies reporting higher prevalences have used alternative detection methods, such as Western Blot or ADVIA-Centaur sIgE measurement (13,21).

Index-reactions to bee stings, in most cases, were highly severe (Mueller grades 3 and 4). Severity of index-reaction appeared to be associated with older age, which has already been supported by previous studies (22) and could be explained by a larger proportion of comorbidities in these patients and its co-factorial influence on reaction severity. However, findings supporting this explanation have been contradicting, and less than a quarter of our patients reported cardiovascular comorbidities, with this variable being non-significant (22,23).

Conversely, another interesting finding is that patients monosensitized to *Api m 1* presented with milder reactions in our multivariate model. Studies assessing sting reactions' severity and *Api m 1* sensitizations have conflicting results.

Api m 1 sIgE levels did not correlate with the severity of index-reactions in previous reports (24,25). However, co-sensitization with *Api m 10* has been linked with severe reactions (21,25), which could help explain why our patients that were not sensitized to both allergens presented with milder systemic reactions.

Only half of our BVA patients underwent VIT. Even though a few patients were contraindicated for it (e.g. pregnancy, active autoimmune diseases), most declined treatment due to not being able to support costs. This has been explained in recent national studies that report the high economic burden that non-reimbursement of immunotherapy has for patients (26).

Sixteen patients (35.6%) reported systemic reactions during immunotherapy, which were globally milder than index-reactions. Although this proportion appears to be higher than in some studies (27,28), it is not largely different than national studies that used similar vaccine manufacturers (29). Treatment protocols and allergen composition could influence the occurrence of reactions during VIT (30). Our patients were treated with a cluster protocol and with aqueous extracts purified from Hymenoptera venom (Roxall Medicina, Spain). Even though sensitization to *Api m 10* appeared to be the single factor in our cohort associated with systemic adverse reactions during VIT, it could subsequently have a protective role in preventing SSR, according to our analysis of re-stung patients. Out of 47 patients undergoing VIT, twenty-five (53.2%) had re-stings. Only four re-stung patients reported SSR – an 84.0% honeybee VIT efficacy, which is in line with the literature (31). It should be noted that only half of our patients were re-stung, stressing the importance of active preventive measures during contact with hymenoptera, particularly in beekeeping activities (e.g. strengthening of body suit protection).

Despite the low number of analyzed patients, some factors related to molecular sensitization were marginally associated with the VIT efficacy. Particularly, *Api m 10* sensitization and higher *Api m 10* sIgE levels could be associated with local

re-sting reactions. This appears to contradict the results of a 2016 study in a Northern European cohort, which hypothesized that their VIT was not enriched with *Api m 10* (32). However, recently published studies in Mediterranean cohort and using similar vaccine manufacturers have hinted at the efficacy of VIT in reducing *Api m 10* levels and the severity of re-stings (20). Additionally, the composition of the Roxall vaccine is known to contain *Api m 10*. This could help explain our results, with patients sensitized to *Api m 10* having systemic reactions during the early stages of VIT but subsequently attaining tolerance to re-stings. Inversely, sensitization to *Api m 4*, despite being observed in very few patients that were also polysensitized, was marginally associated with SSR. *Api m 4* was not detected in the Roxall vaccine composition, which could explain the inefficacy of VIT observed in these patients. Recent studies also appear to corroborate our findings, reporting systemic reactions during VIT and lower efficacy during sting challenge in patients sensitized to *Api m 4* (13).

There are some limitations to be considered in this study. Its retrospective properties and dependency on clinical records could hinder the quality of collected data, especially regarding clinical characteristics such as cardiovascular comorbidities. Secondly, sIgE to molecular components were assessed according to the clinical history and routine diagnosis, but not in a systematic manner in every patient. In particular, *Api m 4* was only recently available, leading to a lack of measurements at baseline, which prevented a deeper multivariate sIgE analysis and a thorough analysis of molecular sIgE. Lastly, the low number of patients that were re-stung has also limited statistical power for potential associations. This should be properly addressed in

prospective studies focused on data gathering, larger cohorts and patient follow-up.

Despite these limitations, we conclude that elderly patients had more severe index reactions, monosensitization to *Api m 1* predicted milder reactions, sensitization to *Api m 10* was associated with a higher likelihood of reactions during VIT but potentially less systemic reactions at re-stings. Molecular sensitization appears to be relevant not only in stratifying the severity of index reactions but also in assessing VIT safety and efficacy. Studies with bigger BVA and VIT cohorts, as well a systematic molecular profiling of patients, are essential to validate these results and improve the clinical and therapeutic approach to BVA.

Fundings: non-financed

Contributions of each author:

JCL: Conceptualization, data curation, formal analysis, investigation, methodology, Writing – original draft, Writing - review & editing

PBA: Conceptualization, data curation, formal analysis, investigation, methodology, Writing – original draft, Writing - review & editing

HPP: Data curation, Writing - review & editing

FC: Data curation, Software, Writing - review & editing

ICF: Data curation**,** writing - review & editing

AM: Methodoloy, Data curation, Supervision and Validation

RC: Methodology, Data curation, Supervision and Validation

GC: Supervision and Validation

AT: Supervision and Validation

BT: Conceptualization, Project administration**,** Supervision and Validation

Conflict of interest: The authors have no conflicts of interest.

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Table I: Characteristics of patients with bee sting reactions (n=93)

Variables	Values	Total patients
Demographic data		
Current age	46 (34-55)	93
Male gender, n (%)	52 (55.9)	93
Allergic comorbidities, n (%)	43 (57.3)	75
Asthma, n (%)	15 (22.4)	67
Rhinitis, n (%)	30 (43.5)	69
Food allergy, n (%)	4 (9.1)	44
Cardiovascular comorbidities, n (%)	18 (23.4)	77
Beekeeper, n (%)	40 (43.0)	93
Clinical Data – index-reaction		
Age at reaction, M (IQR)	36 (26-48)	68
Severity classification (Müeller), M (IQR)	3 (2-3)	85
Grade 1, n (%)	12 (14.1)	85
Grade 2, n (%)	20 (23.5)	85
Grade 3, n (%)	35 (41.2)	85
Grade 4, n (%)	18 (21.2)	85
Number of stings in the same reaction, n (%)		
Once, n (%)	30 (75.0)	40
Twice, n (%)	4 (10.0)	40
Three or more times, n (%)	6 (15.0)	40
Clinical data – specific immunotherapy		
Patients undergoing VIT, n (%)	47 (50.5)	93
Completed treatment (median: 5 years), n (%)	19 (40.4)	47
Discontinued treatment, n (%)	2 (4.2)	47
Under treatment, n (%)	26 (55.4)	47
Adverse reactions during VIT, n (%)	16 (35.6)	45
Re-stung patients, n (%)	25 (53.2)	47
Systemic reactions, n (%)	5 (19.2)	26
Severity classification (Müeller)	2 (1-2)	5
Laboratory data		
Basal tryptase	4.2 (3.4-5.6)	72
Basal tryptase >11.4 ug/L, n (%)	3 (4.2)	72
Apis melífera IgE (total)	6.7 (1.0-20.3)	93
Apis mellifera IgE (VIT: pre-treatment)	13.7 (5.3-43.5)	47
Apis mellifera IgE (VIT: >12M treatment)	3.3 (1.1-7.9)	42
Api m 1 IgE	0.5 (0.1-1.6)	73
Positive (>0.34 kU/L), n (%)	42 (57.5)	73
Api m 4 IgE	0.01 (0.0-0.08)	42
Positive (>0.34 kU/L), n (%)	5 (11.9)	42
Api m 10 IgE	0.3 (0.1-1.6)	64
Positive (>0.34 kU/L), n (%)	32 (50.0)	64
Api m 1 (+) / 4 (-) / 10 (-), n (%)	5 (11.9)	42
Api m 1 (-) / 4 (+) / 10 (-), n (%)	0 (0.0)	42
Api m 1 (-) / 4 (-) / 10 (+), n (%)	2 (4.8)	42
Polysensitized *Api m 1/4/10 (+)*, n (%)	3 (7.1)	42

Legend: IQR – interquartile range; M – median.

Table II: Associations between the variables analyzed and the severity of the index-reaction (n=85)

Variables	Reaction severity grading (Müeller)				Total patients	*p-value*	Spearman Coefficient
	1 (n=12)	2 (n=20)	3 (n=35)	4 (n=18)			
Male gender, n (%)	7/12 (58.3)	11/20 (55.0)	21/35 (60.0)	9/18 (50.0)	85	*0.782*	
Age at time of index-reaction, M (IQR)	28.0 (12.0-40.0)	35.5 (27.0-45.5)	40.0 (29.0-53.0)	40.5 (28.5-55.0)	68	***0.040***	0.249
Allergic comorbidities, n (%)	5/11 (45.4)	7/13 (53.8)	18/30 (60.0)	11/14 (78.6)	68	*0.090*	
Cardiovascular comorbidities, n (%)	1/11 (9.1)	4/18 (22.2)	8/28 (28.6)	4/15 (26.7)	72	*0.308*	
Beekeepers, n (%)	5/12 (41.7)	7/20 (35.0)	16/35 (45.7)	7/18 (38.9)	85	*0.849*	
Basal tryptase, M (IQR)	5.2 (3.6-5.9)	3.7 (2.8-4.4)	4.2 (3.4-5.7)	5.0 (4.0-5.6)	64	*0.179*	0.170
Apis mellifera IgE (kU/L), M (IQR)	6.4 (0.01-12.4)	5.3 (1.1-10.6)	10.6 (1.9-42.8)	2.8 (0.4-34.7)	83	*0.409*	0.092
Api m 1 IgE (kU/L), M (IQR)	0.3 (0.02-1.3)	0.5 (0.2-1.3)	0.5 (0.1-1.6)	0.5 (0.01-2.1)	70	*0.804*	0.030
Positive (>0.34 kU/L), n (%)	5/11 (45.4)	12/17 (70.6)	14/25 (56.0)	9/17 (52.9)	70	*0.856*	
Api m 4 IgE (kU/L), M (IQR)	0.39 (0.01-0.77)	0.01 (0.01-0.02)	0.0 (0.0-0.04)	0.04 (0.01-0.18)	41	*0.734*	0.055
Positive (>0.34 kU/L), n (%)	1/2 (50.0)	1/10 (10.0)	1/17 (5.9)	2/12 (16.7)	41	*0.869*	
Api m 10 IgE (kU/L), M (IQR)	1.06 (0.23-1.31)	0.24 (0.07-0.44)	0.41 (0.11-1.99)	0.14 (0.02-2.59)	62	*0.580*	-0.072
Positive (>0.34 kU/L), n (%)	4/6 (66.7)	7/16 (43.8)	13/25 (52.0)	7/15 (46.7)	62	*0.765*	
Api m 1 (+) / 4 (-) / 10 (-), n (%)	1/2 (50.0)	3/10 (30.0)	1/17 (5.9)	0/12 (0.0)	41	***0.015***	
Api m 1 (-) / 4 (+) / 10 (-), n (%)	NA	NA	NA	NA	41	*NA*	
Api m 1 (-) / 4 (-) / 10 (+), n (%)	0/2 (0.0)	1/10 (10.0)	1/17 (5.9)	0/12 (0.0)	41	*0.658*	
Polysensitized *Api m 1/4/10 (+)*, n (%)	1/2 (50.0)	0/10 (0.0)	1/17 (5.9)	1/12 (8.3)	41	*0.736*	

Legend: M – median; IQR – interquartile range

Table III: Associations between the variables analyzed and the proportion of patients with reactions during VIT (n=45)

Variables	Reactions during VIT (n=45)		Total patients	*p-value*
	Yes (n=16)	No (n=29)		
Male gender, n (%)	10/16 (62.5)	20/29 (69.0)	45	*0.660*
Age at time of most severe reaction, M (IQR)	38 (31-42)	30 (21-37)	25	*0.113*
Severity of most severe reaction, M (IQR)	3 (3-4)	3 (2-3)	37	*0.142*
Allergic comorbidities, n (%)	6/13 (46.2)	9/25 (36.0)	38	*0.544*
Cardiovascular comorbidities, n (%)	1/14 (7.1)	1/19 (5.3)	33	*0.999*
Beekeeper, n (%)	11/16 (68.8)	16/29 (55.2)	45	*0.373*
Basal tryptase, M (IQR)	4.4 (3.1-5.0)	4.3 (3.5-5.7)	37	*0.340*
Apis mellifera IgE (pre-treatment), M (IQR)	16.4 (6.4-32.2)	12.4 (3.3-50.1)	45	*0.847*
Apis mellifera IgE (>12M treatment), M (IQR)	2.6 (1.1-8.0)	3.8 (0.6-6.7)	37	*0.808*
Api m 1 IgE (kU/L), M (IQR)	0.6 (0.4-1.0)	1.1 (0.3-2.9)	25	*0.397*
Positive (>0.34 kU/L), n (%)	8/10 (80.0)	10/15 (66.7)	25	*0.550*
Api m 4 IgE (kU/L), M (IQR)	0.04 (0.01-0.10)	0.01 (0.0-0.14)	19	*0.350*
Positive (>0.34 kU/L), n (%)	1/8 (12.5)	1/11 (9.1)	19	*0.999*
Api m 10 IgE (kU/L), M (IQR)	1.4 (0.5-2.6)	0.3 (0.2-2.9)	23	*0.418*
Positive (>0.34 kU/L), n (%)	9/10 (90.0)	6/13 (46.2)	23	***0.038***
Api m 1 (+) / 4 (-) / 10 (-), n (%)	0/8 (0.0)	2/9 (18.2)	19	*0.485*
Api m 1 (-) / 4 (+) / 10 (-), n (%)	NA	NA	NA	*NA*
Api m 1 (-) / 4 (-) / 10 (+), n (%)	1/8 (12.5)	0/11 (0.0)	19	*0.421*
Polysensitized *Api m 1/4/10 (+)*, n (%)	1/8 (12.5)	1/11 (9.1)	19	*0.999*

Legend: IQR – interquartile range; M – median.

Table IV: Associations between the variables analyzed and the proportion of patients with systemic reactions to re-stings (n=25)

Variables	Systemic reactions to re-stings (n=25)		Total patients	*p-value*
	Yes (n=4)	No (n=21)		
Male gender, n (%)	2/4 (50.0)	15/21 (71.4)	25	*0.660*
Age at time of most severe reaction, M (IQR)	28 (27-29)	36 (29-42)	11	*0.158*
Severity of most severe reaction, M (IQR)	3 (3-4)	3 (2-3)	21	*0.142*
Allergic comorbidities, n (%)	1/1 (100.0)	4/16 (25.0)	17	*0.100*
Cardiovascular comorbidities, n (%)	0/3 (0.0)	1/13 (7.7)	16	*0.999*
Beekeeper, n (%)	3/4 (75.0)	15/21 (71.4)	25	*0.999*
Basal tryptase, M (IQR)	3.0 (2.05-3.7)	4.25 (3.6-4.8)	22	*0.055*
Apis mellifera IgE (pre-treatment), M (IQR)	6.18 (3.3-37.61)	13.7 (5.89-41.2)	25	*0.543*
Apis mellifera IgE (>12M treatment), M (IQR)	4.28 (2.82-71.4)	1.97 (0.61-8.32)	23	*0.268*
Api m 1 IgE (kU/L), M (IQR)	1.02 (0.39-22.34)	0.54 (0.42-1.18)	12	*0.397*
Positive (>0.34 kU/L), n (%)	7/8 (87.5)	3/4 (75.0)	12	*0.999*
Api m 4 IgE (kU/L), M (IQR)	1.66 (0.00-3.14)	0.03 (0.01-0.14)	9	*0.350*
Positive (>0.34 kU/L), n (%)	2/3 (66.7)	0/6 (0.00)	9	*0.087*
Api m 10 IgE (kU/L), M (IQR)	0.15 (0.14-0.17)	1.08 (0.37-2.63)	11	*0.059*
Positive (>0.34 kU/L), n (%)	1/4 (25.0)	6/7 (85.7)	11	*0.093*
Api m 1 (+) / 4 (-) / 10 (-), n (%)	0/3 (0.0)	2/6 (33.3)	9	*0.500*
Api m 1 (-) / 4 (+) / 10 (-), n (%)	NA	NA	NA	*NA*
Api m 1 (-) / 4 (-) / 10 (+), n (%)	1/6 (16.7)	0/3 (0.0)	9	*0.999*
Polysensitized *Api m 1/4/10 (+)*, n (%)	2/3 (66.7)	0/6 (0.00)	9	*0.087*