IgE-Api m4 is useful to identify a particular phenotype of allergy to bee venom.

Short title: Allergy to Api m 4 from bee venom.

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Abstract

Background: Different clinical behaviours have been identified in patients allergic to bee venom. Compound resolved diagnosis could be appropriated to looking for these differences. Objective: to analyse if sIgE-Api m 4 is able to identify a particular kind of allergy to bee venom, and to describe the response to bee venom immunotherapy (bVIT) of patients.

Methods: Prospective study including 31 patients allergic to bee venom, who were allocated depending on sIgE-Api m4<0.98 kU/L in phenotype-A, receiving native aqueous (NA) extract sIgE-Api m4 ≥0.98 kU/L in phenotype-B, receiving purified aqueous (PA) extract. Gender, age, cardiovascular risk, grade of preceding sting reaction, beekeeping, and immunological data (Intradermal test, sIgE and sIgG4-Apis-nApi m1, and sIgE-rApi m2-Api m4 were analyzed. Systemic reactions (SRs) during bVIT induction were analyzed. Immunological and sting challenge outcomes were evaluated after 1 and 2 year of bVIT in two separate phenotypes.

Results: Phenotype-B patients showed more severe reactions (*P*=.049) and higher skin sensitivity (*P*=.011), baseline sIgE-Apis (*P*=.0004), sIgE-nApi m1 (*P*=.0004), and sIgG4-Apis (*P*=.027) than phenotype-A ones. Furthermore, 41% of phenotype-B patients suffered SRs during NA updosing with a sting challenge success of 82%. Serial intradermal test did not significantly decreased but an intense reduction in sIgE-nApi m1 (*P*=.013) and sIgE-Api m4 (*P*=.004) were confirmed since the first year of bVIT.

Key Words: bee venom allergens; bee venom immunotherapy; component resolved diagnosis; sting challenge; systemic reaction.

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Resumen

Antecedentes: Los pacientes alérgicos a veneno de abeja muestran distintos comportamientos clínicos. El diagnóstico por componentes podría ayudar a entenderlos. Objetivo: estudiar la capacidad de la IgE-Api m4 para identificar diferentes patrones de alergia al veneno de abeja, incluyendo la respuesta a la Inmunoterapia al veneno de abeja (ITVa).

Métodos: Estudio prospectivo de 31 pacientes alérgicos al veneno de abeja, distribuidos en dos grupos fenotípicos (A y B) en función de sus niveles de IgE-Api m4 (punto de corte 0.98kU/L) y tratados con extracto acuoso nativo (AN)-fenotipo A, o extracto acuoso purificado (AP)-fenotipo B. Se analizaron sexo, edad, riesgo cardiovascular, gravedad de la picadura, exposición, y datos inmunológicos (intradermorreación, IgE e IgG4-Apis-nApi m1 e IgE-rApi m2-Api m4). Se analizó la seguridad en la fase de inicio de la ITVa, y la eficacia y cambios inmunológicos después de 1 y 2 años de ITVa.

Resultados: El fenotipo-B mostró reacciones más graves con las picaduras (p=.049), una mayor sensibilidad cutánea (p=.011) y valores más elevados de IgE-Apis (p=.0004), IgE-nApim1 (p=.0004), e IgG4-Apis (p=.027) que el fenotipo-A. Por otra parte, 41% de los pacientes del fenotipo-B sufrió reacciones sistémicas durante el inicio con AN, con una tasa de protección del 82%. La respuesta cutánea no mejoró significativamente, y se comprobó la reducción intensa de IgE-nApi m1 (p=.013) e IgE-Api m4 (p=.004) desde el primer año de ITVa.

Conclusión: El uso de la IgE-Api m4 como único criterio discriminativo ha podido confirmar que hay diferentes maneras de ser alérgico al ITVa. Se necesitan estudios en poblaciones más amplias.

Palabras clave: alérgenos del veneno de abeja; inmunoterapia con veneno de abeja; diagnóstico por componentes; repicadura; reacción sistémica.

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INTRODUCTION

It is well known that bee venom immunotherapy (bVIT) is associated with increased risk of systemic reactions (SRs) and decreased protection against bee sting, compared to vespid venom immunotherapy [1,2].

To date, 12 different allergens have been identified in bee venom [3]. The most common is phospholipase A2 (Api m1), independent from the method used to detect specific IgE (sIgE) [4–6]. Hyaluronidase (Api m2) is a cross-reactivity marker [7]. Melittin (Api m4) is a 2.84 kDa peptide found in abundance in the venom [8], to which a low allergenicity is attributed [4]. Joint use of these three components, which make up the bulk of the dry weight of the venom and they are the best known so far, has proven to increase diagnostic capability by 15% compared to the diagnosis conducted with rApi m1 exclusively [9]. A later study showed the complexity of different sensitisation profiles in patients allergic to bee venom, using up to 6 individual components [4].

A recent study found that a high number of patients allergic to bee venom show a high prevalence of sensitisation to Api m4 among patients who suffered SRs during induction of bVIT, therefore the incidence of SRs was associated with sensitization to Api m 4 [10].

The other hand, allergenic extracts currently available for bVIT, are standardised on the basis of their enzymatic activity and total protein content, with no consideration of individual components. Taking both aspects into consideration, the question should be whether there are different profiles of patients allergic to bee venom that require a more specific diagnosis according to the determination of individual allergenic components, instead of conventional diagnosis on the basis of sIgE to full extracts (intradermal test

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(IDT) or serum sIgE), in order to carry out a therapeutic approach adapted to such sensitisation profile.

This study intends to analyse if sIgE to Api m 4 is able to identify a particular kind of allergy to bee venom, in terms of clinical and sensitization profile as well as to describe the response to bVIT of patients.

METHODS

Study design:

Longitudinal prospective study of patients suffering anaphylaxis due to bee sting, diagnosed by conventional and molecular tools and treated with bVIT, after giving written informed consent to the protocol approved by the Clinical Research Ethics Committee of Reina Sofia University Hospital (Córdoba, Spain).

Demographics and clinical data:

Initially collected data were demographics: gender, age, link with beekeeping (yes/no); clinic: grade of anaphylaxis (I-IV) after sting according Müller classification [11] and cardiovascular risk (coronary disease, hypertension, and β-blockers or angiotensin-converting enzyme inhibitor treatment).

Conventional diagnosis:

We performed serial IDT with *Apis mellifera* venom (ALK Abelló SA, Madrid, Spain) using increasing concentrations from 0.00001 to 0.1 µg/ml. The lowest concentration to produce a wheal measuring 5 mm in mean diameter on average was considered positive.

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Serum sIgE, and specific IgG4 (sIgG4) to Apis, were determined using the ImmunoCAP technology (Thermo Fisher Scientific, Uppsala, Sweden) according to manufacturer's instructions. Quantitative results were expressed in kU/L (sIgE) and µg/mL (sIgG4**).**

Basal serum tryptase levels were measured using ImmunoCAP Tryptase (Thermo Fisher Scientific, Uppsala, Sweden).

Molecular diagnosis:

sIgE to molecular components of *Apis mellifera* venom: nApi m1 (Sigma-Aldrich, Madrid, Spain), rApi m2 expressed in purified cells infected with baculovirus [7], and Api m4 a synthetic peptide (Schafer-N, Denmark) was determined using the ADVIA-Centaur system (Bayer Health Care Diagnostics Division, Tarrytown, New York, USA) and expressed in kU/L [12].

sIgG4 to Api m 1 (µg/mL) was determined using the ImmunoCAP technology (Thermo Fisher ScientificUppsala, Sweden).

Patients:

Patients diagnosed of allergy to bee venom after suffering anaphylaxis due to bee sting were allocated depending on their baseline value of sIgE to Api m4 in phenotype A (IgE<0.98 kU/L) or phenotype B (IgE≥0.98 kU/L). In absence of validated reference, the cut-off 0.98 kU/L was decided as the median value resulting from a pilot sample of 25 biobank sera with sIgE to Api m4 detectable.

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Immunotherapy:

Immunotherapy was indicated according to the international guidelines [13] and administered in an immunotherapy unit by the same nursing staff, trained and experienced in recognising and treating anaphylactic reactions, under direct supervision of an allergist.

The following extracts were used for treatment: the native aqueous extract (NA) (Pharmalgen®, ALK-Abelló SA, Madrid, Spain) and purified aqueous extract (PA) (Aquagen®, ALK-Abelló SA, Madrid, Spain) of bee venom. According the manufacturer information, PA venom extracts are products with scarce amount of low-molecular components present in the native venom extract, which should include a depletion of Api m 4. Patients with phenotype A were treated with PA and patients with phenotype B were treated with NA, aiming a better adequacy among the sensitization profiles and the allergenic content of the extracts.

Updosing phase was performed in all patients according to the same clustered protocol previously described [14]. Maintenance was decided with 200 μg in the case of patients under higher exposure bee stings, and 100 μg in the rest. Monthly injections were given along two years.

Evaluation of bVIT Safety:

SRs (yes/no) and number SRs during induction of immunotherapy, were reported according to the Müller classification previously used for diagnosis [11].

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All our patients began bVIT without premedication, in order to avoid a confounding influence of premedication on the absence of SRs. In the case of an initial SR occurrence, pretreatment with Dexchlorpheniramina (5mg) and Methylprednisolone (1mg/kg) will be prescribed from the next appointment on; the dose was not reduced.

Evaluation of sting challenge:

An in-hospital sting challenge test with a living honeybee was offered to all patients who reached the maintenance dose and had no SRs, for evaluation of efficacy of treatment. The test was carried out after 1 and 2 years of immunotherapy, as previously described with vespids [15]. The response was classified according to Muller [11]. When the challenge was negative, the patients remained under observation for 2 hours after the sting challenge.

Evaluation of Immunologic Markers

IDT response was measured after 1 and 2 years of immunotherapy and grouped according their concentration (0.0001-0.1, 0.1 y ≥1 µg/ml) for analysing. The lowest concentration to produce a weal of 5 mm diameter on average was recorded as positive and a reduction in concentration eliciting a positive response was evaluated as IDT improvement.

Serum sIgE and sIgG4 to Apis, and sIgE nApi m1-rApi m2-Api m4 and sIgG4 to Api m1 were determined, after 1 and 2 years of immunotherapy. All immunological data were analysed in two separate phenotypic groups (A and B).

Statistical Methods

The description of quantitative values was performed using the mean, standard

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deviation, median, interquartile range and minimum and maximum values. The t of Student's t distribution and Mann-Whitney U test were applied for quantitative variables. The chi-square test, Fisher test, Cochran-Armitage and Symmetry test were applied to examine the independence between qualitative variables. To find relation between quantitative variables, we used Spearman's rank correlation coefficient. All analyses were performed with SAS statistical software version 9.3.

RESULTS

1. PATIENTS AND SENSITISATION PROFILE

A total of 31 patients were included; Table 1 collects their demographic, clinical, and diagnostic characteristics. Figure 1 shows the population flow chart.

According to the cut-off 0.98 kU/L for sIgE to Api m4, 19 patients were assigned to group A and 12 to group B, conforming two phenotypes that include differences in the sting reaction severity, intradermal reaction intensity, and baseline sIgE levels to Apis and nApi m1, and baseline sIgG4 levels to Apis (Table 1). Figure 2 shows the sensitisation profiles of both phenotypes.

2.- RESULTS IN PHENOTYPE A:

2.1 - Clinical Changes

2.1.1 bVIT Safety

All of the 19 patients were treated with PA extract. Thirteen reached the 100 μg maintenance dose and six the 200 μg dose. Three patients suffered two Müller grade I SR during the updosing of bVIT; the second SR in each patient occurred despite

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premedication. After completing the updosing phase, one patient withdrew, due to reasons unrelated to the study.

2.1.2 Sting challenge outcomes.

The 18 patients in this group tolerated a sting challenge test after one and two years of bVIT.

2.2 Immune Changes

Table 2 shows the progress of sIgE, sIgG4 values, and IDT response comparing the determinations at the end of the first and second year, respectively, with the baseline values.

3.- RESULTS IN PHENOTYPE B:

3.1 - Clinical Changes

3.1.1 bVIT Safety

All of the 12 patients were treated with NA extract. Four reached the 100 μg maintenance dose and eight the 200 μg dose. At the start of bVIT, three patients showed 1 SR (2 with Müller grade I and 1 with Müller grade III). Two patients showed 17 episodes, 15 of them despite premedication (6 with Müller grade I and 11 with Müller grade III). A patient continued to show SRs during maintenance. After completing the updosing phase, one patient from the group B withdrew, due to reasons unrelated to the study.

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3.1.2 Sting challenge outcomes.

A patient from this group was not subject to the sting challenge test due to constant SRs during maintenance, and was accounted for as a therapy failure (intention to treat). Another single patient showed grade III SR both in the first and second years. Efficacy in this group is 82%.

3.2 Immune Changes

Likewise, Table 2 shows the progress data for the immune markers (sIgE, sIgG4, IDT) for phenotype B.

DISCUSSION

We integrated in our daily clinical to the regular clinical practice a tool based on the compound resolved diagnosis (CRD), seeking phenotypic differences that allow for better handling of bee venom allergic patients, including the possibility of using immunotherapy safety and efficacy biomarkers.

Among patients allergic to bee venom, Api m1 acts as the dominant sensitizer when using both the natural antigen [6] and the recombinant [4,5]. In this study, nApi m1 was recognised by 100% of patients from both phenotypes on the basis of a cut-off point of 0.35 kU/L. However, prevalence of sensitisation of rApi m2 in our population (52.6% in phenotype A, and 75% in phenotype B) is greater than that described by other authors (47.9% by Köhler [4], and 52.2% by Sturm [9], which may be justified by regional differences or sensitising sources other than bee venom, as Api m2 is recognised as a cross-reactivity marker between bee and wasp venoms [16]. The third allergen studied, Api m4 synthetic protein, has shown a 47% prevalence in phenotype A, similar to that

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described in a recent work [9]. Contemplating that the recognition of Api m 4 is 100% in phenotype B, the Api m 4 is considered a major allergen in our population. This confirms the findings of a previous study [10]. On a global scale, these data suggests that phenotype A patients have a sensitisation profile similar to that described by other authors, while phenotype B patients show a high rate of polysensitisation.

Additionally, all patients sensitised to Api m4 are also sensitised to nApi m1 (0.35 kU/L) and some patients also to rApi m2, which leads us to think that sIgE to Api m4 could act as a marker of "advanced allergic march", representing a more complex form of the disease due to a different sensitisation pattern.

Using the sIgE-Api m4 as the unique discrimination criteria (based in an arbitrary cuttoff point in absence of knowledge about the true potential of Api m 4 to generate IgE response when the protocol was decided), we have found that patients from group B had more serious SR after the stings, showing a higher baseline skin sensitivity and higher sIgE levels to full Apis venom and to nApi m1. Also, higher baseline sIgG4-Apis levels were found, suggesting the possibility of a higher number of prior stings as described by Müller [17], despite the ratio of beekeepers and the age of the subjects is similar between phenotypes A and B.

Interestingly, all this leads to the interpretation that phenotype B is a more complex or possibly more advanced form of the disease.

We found a high SRs incidence with bVIT in some phenotype B patients, which could be explained by individual predisposition factors (namely, higher levels of sIgE-Apis, sIgE-nApi m1, and sIgE-Api m4), thus strengthening the value of Api m4 as a witness of poor tolerance to bVIT, or secondly by factors in the NA extract [18]. Tryptase levels

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were normal and the updosing schedule was previously demonstrated as safe, using the same extract [14]; therefore, they were ruled out as factors associated to poor VIT tolerance in a multicenter trial [19].

Known bVIT-induced immune changes [20,21], measured in decrease percentages of sIgE-Apis levels and increase percentages of sIgG4-Apis,were confirmed in both phenotype A and phenotype B. A similar behaviour can be seen in the sIgE-nApi m1 and sIgG4-Api m1 levels and similar results were described for sIgE-nApi m1 [22] and sIgG4-Api m1 [4].

The values of sIgE-Api m4 decreased significantly in phenotype B since the first year of bVIT, something not found in phenotype A. This phenomenon in phenotype A can be explained by the minimum levels at the beginning of the treatment and the depletion of the part containing Api m4 in the PA extract. It would have been of interest to know the IgG4-Api m4 levels to confirm the response to immunotherapy at this level, something already proven by Köhler in a group of 20 patients, although without specifying what type of extract they were treated with [4]. This tool can indirectly address the normal lack of knowledge about the regular allergenic contents in hymenoptera venom vaccines, due to their special standardisation mode.

Skin sensitivity in phenotype B patients increased at baseline and was not significantly reduced after one year of immunotherapy. A similar effect was previously published [23], and it has been proven that change is obvious after 5 years of treatment [21,22]. Patients from phenotype A show reduced skin reactivity from the first year forward, which suggests a difference affecting the inflammatory parameters of skin mast cells, which may be altered by immunotherapy apart from changes in serum immunoglobulin.

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In some cases, spontaneous field stings may provide information confirming clinical protection during or after VIT. However, spontaneous stings are always unpredictable, as they can inject a low venom quantity and the response is never verified.

In our study, patients received a controlled challenge sting 1 and 2 years after continuous bVIT, in order to confirm their protection, as it has been described in a yellow jacket population that a single tolerated re-stinging cannot ensure maximum protection [24]. In our study, phenotype A patients reached protection in 100% of cases in the first year, sustained in the second year, using the same purified extract where a Ruëff series failed in 15% of cases [20]. The authors attribute this to insufficient dosage for this patient subgroup of unknown phenotype. Phenotype B patients reached a protection of 82% after the first year of treatment, and did not improve during the second. Two patients represent the remaining 18%; one who was not subject to a re-stinging (patient suffered spontaneous stings with milder systemic reactions than prior bVIT began), and other who showed early cervico-facial erythema and wheezing after two in hospital re-stingings, that responded properly to adrenalin. After the first positive sting, dose was increased to 200 μg/4 weeks, and to 300 μg/4 weeks after the second. Both patients suffered repeated SRs during bVIT start, and the first also during maintenance, confirming the therapy failure prediction value, having poor tolerance to bVIT [2].

Protection of 93% of the global population, even higher to that obtained in other series [2,25], invites to allocate patients using the proposed criteria so as to achieve an adequate selection of extracts. A review in 2010 indicated that the efficacy of purified and non-purified (native) bee venom extracts is similar, although possible patient

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variability was not taken into account, as found in this work through a specific design for this purpose [26].

Despite the possible limitations in sample size and in the number of available allergens, the present pilot study allows us to confirm that there are different ways to be allergic to bee venom. During clinical practice we could identify a sub-population of patients who suffered anaphylactic reactions including respiratory or hemodynamic instability after stings, showed high skin sensitivity, sIgE to Api m4 levels >0.98 kU/L and were predisposed to show SRs with bVIT. We called this group “phenotype B” and treated its subjects with a full bee venom extract, with a success of 82% of cases. During follow up we confirmed a special difficulty in negativising papule, as well as an intense reduction in sIgE levels to nApi m1 and Api m4. The response to rApi m2 was not eloquent. This phenotype includes patients that might benefit from specific interventions from the beginning, such as premedication during bVIT, already proposed to achieve better tolerance [27–29], different updosing schedules, or the use of higher venom doses [30].

The results of the current study encourage properly designed multicentre studies that include wide populations and explore new chances of CRD and therapeutic option on the basis of sensitisation profiles found, and their use as eventual severity and treatment response markers, thus increasing the therapeutic benefits and improving the risk-benefit balance for patients who suffer this life-threatening disease.

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Table 1 Characteristics of both population groups of patients allergic to the venom of *Apis mellifera.*

CARACTERISTIC	PHENOTYPE A	PHENOTYPE B	*P* value
Patients, n. (%)	19 (61.3)	12 (38.7)	
Male patients, n. (%)	15 (79)	11 (91.6)	0.633
Age, mean (SD), year	38 (16.4)	36.4 (19)	0.787
Beekeepers, n. (%)	10 (52.6)	8 (66.6)	0.484
Pesence Cardiovascular risk[a], n. (%)	3 (15.7)	2 (16.6)	1.000
Bee-Sting Reaction Grade[b],n. (%)			
I	3 (15.7)	2 (16.6)	
II	8 (42)	0	0.049
III	7 (36.8)	6 (50)	
IV	1 (5.2)	4 (33.3)	
sIgE *Apis mellifera*, median (IQR), kU/L	4.3(11.5)	35 (52.6)	0.0004
Intradermal reaction test, n. (%)			
0.0001µg/ml	0	3 (25)	
0.001µg/ml	6 (31.5)	5 (41.6)	0.011
0.01µg/ml	1 (5.2)	2 (16.6)	
0.1µg/ml	7 (37)	1 (8.3)	
≥1µg/ml	5 (26.3)	1 (8.3)	
Basal Tryptase, median (IQR), µg/L	5.48 (4.4)	5.27 (3)	0.584
sIgE nApi m 1, median (IQR),kU/L	5.12 (12)	55.2 (77)	0.0004
sIgE rApi m 2, median (IQR), kU/L	1.52 (7)	5.5 (127.5)	0.221
sIgE Api m 4, median (IQR), kU/L	0.3 (0.5)	2.7 (8.2)	0.0001
sIgG4 *Apis mellifera*, median (IQR), µg/mL	698 (1525)	2235 (6590)	0.027
sIgG4 Api m 1, median (IQR), µg/mL	1.2 (1.9)	3.3 (3.5)	0.096

sIgE, specific IgE; sIgG4, specific IgG4; IQR, interquartile range.
[a]Cardiovascular risk was considered coronary disease, hypertension, and β-blockers or angiotensin-converting enzyme inhibitor treatment.
[b]The field bee-sting reactions were classified according to the classification of Müller.

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Table 2 Evolution of Inmune changes in Phenotype A and B.

	PHENOTYPE A (n=18)					PHENOTYPE B (n=11)				
	Baseline	1 Year	*P* value[a]	2 Year	*P* value[b]	Baseline	1 Year	*P* value[a]	2 Year	*P* value[b]
sIgEApis,median (IQR), kU/L	4 (11.5)	2 (5.6)	0.038	2 (4.8)	0.02	42 (63.5)	15 (31)	0.001	13.4 (14.2)	0.001
IDT Improvement[c], n.	_	9	0.02	9	NS	_	4	NS	4	NS
sIgEnApi m1, median (IQR), kU/L	5.2 (11.9)	3 (6.2)	0.02	2.3 (5.3)	0.01	63.3 (92.8)	20.6 (32.5)	0.013	23 (39.7)	0.002
sIgErApi m2, median (IQR), kU/L	1.55 (6.9)	5.3 (10.3)	NS	3.9 (10.5)	NS	9 (162.6)	3.5 (44)	NS	4.33 (33)	NS
sIgE Api m4, median (IQR), kU/L	0.33 (0.5)	0.29 (0.4)	NS	0.25 (0.3)	NS	3.7 (8.7)	1.2 (3.1)	0.004	0.7 (1.4)	0.002
sIgG4 Apis, median (IQR), µg/mL	688 (1519)	5130 (6210)	0.001	4378 (5721)	0.001	2213 (6582)	10522 (9734)	0.001	12436 (15049)	0.002
sIgG4 Api m 1, median (IQR), µg/mL	1.3 (2.4)	4.1 (6)	0.0001	5.4 (6)	0.0001	3.1 (3.4)	8.5 (7)	0.001	7.7 (6.7)	0.001

sIgE, specific IgE; IDT, intradermal test; sIgG4, specific IgG4; IQR, interquartile range; NS, not significative.

[a]P-value resulting from the comparison of data at the end of the first year with the baseline values.

[b]P-value resulting from the comparison of data at the end of the second year with the baseline values.

[c]IDT improvement was considered as reduction in concentration eliciting a positive response.

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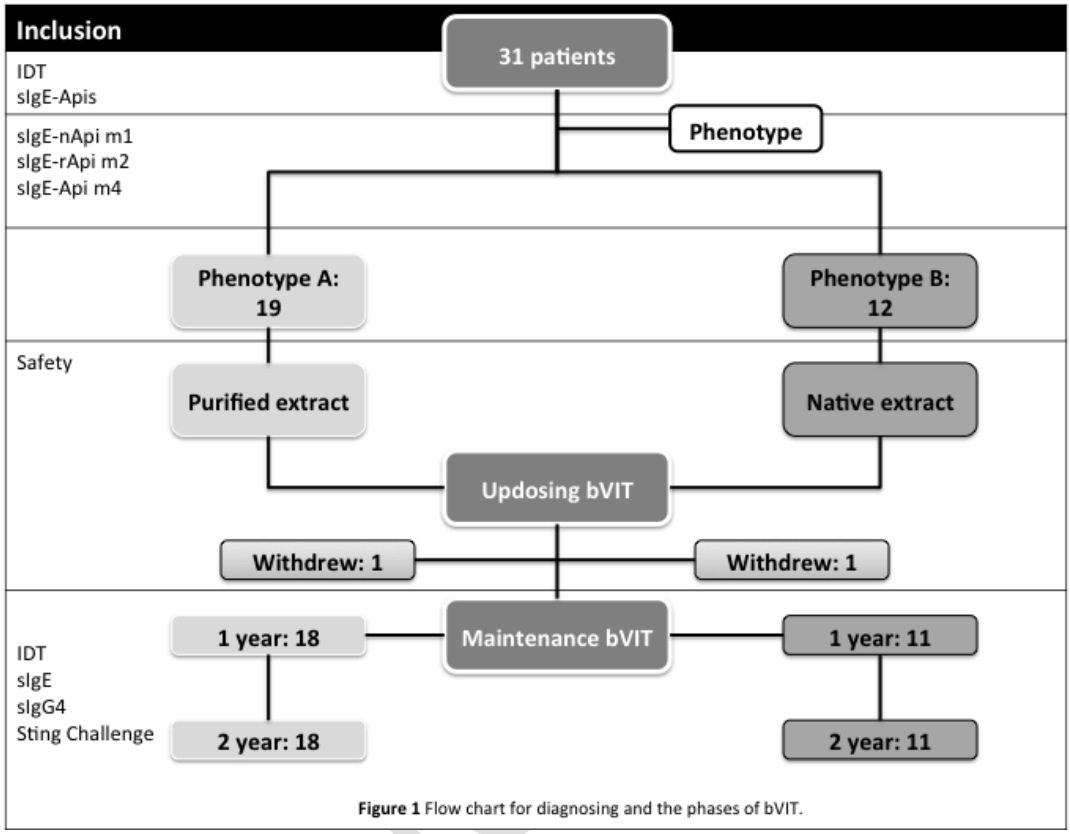
Figure 1 Flow chart for diagnosing and the phases of bVIT.



Legend Figure 1: IDT, intradermal test.

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Figure 2 Sensitization profile (%) in both phenotypes.

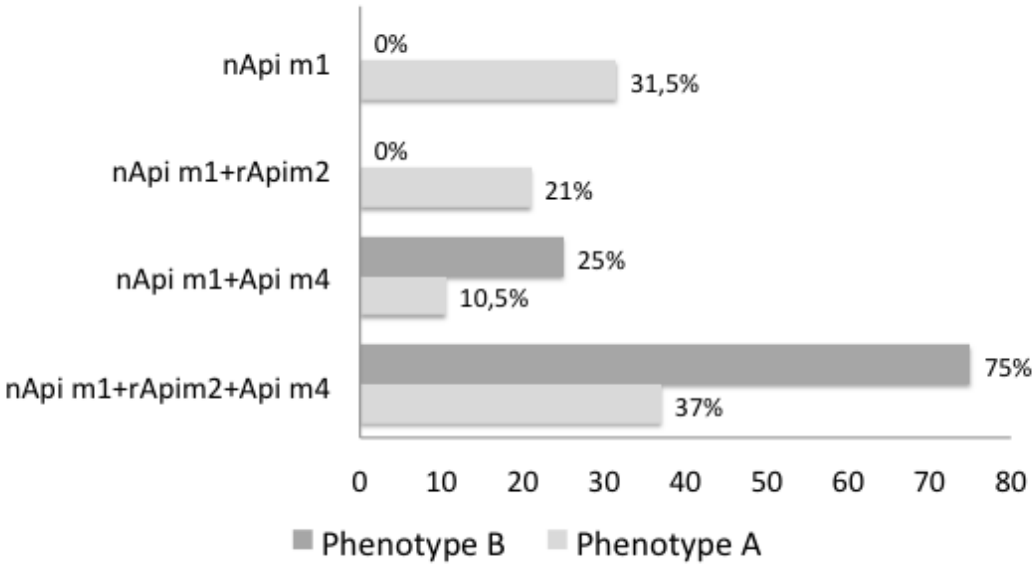

Figure 2 Sensitization profile (%) in both phenotypes.

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