Diplomarbeit

Negative predictive value of the basophil activation test

Beatrix Gertrude Pickl-Herk

Geb.Dat.: 02.02.1984

zur Erlangung des akademischen Grades

Doktorin der gesamten Heilkunde

(Dr. med. univ.)

an der

Medizinischen Universität Graz

ausgeführt an der

Universitätsklinik für Dermatologie und Venerologie

LKH – Univ. Klinikum Graz

unter der Anleitung von

Ass.-Prof. Priv.-Doz. Dr.med.univ. Gunter Sturm

Graz, 23.09.2012

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Acknowledgment

Writing this thesis would not have been possible without the support of several people:

First of all I want to thank my supervisor Ass.Prof. Priv.-Doz. Dr.med.univ. Gunter Sturm, for piquing my interest in allergology during his classes and for his support and advice especially during the final phase of my work. I also want to thank Dr. Danijela Bokanovic with whom I worked closely together throughout the whole study for her help and advice. I very much enjoyed working together with her. I want to thank Karin Loipold for teaching me how to perform the basophil activation test and for making me feel welcome whenever I "invaded" her working space.

Most importantly I want to thank my family. First of all my parents, for their financial support, without which attending medical school would not have been an option for me. I want to thank them especially for their moral support throughout my whole studies and particularly during tough phases. I want to thank my brother who often reminds me not to be such a perfectionist. Last but not least I want to thank my sister for her moral support and her advice, which is invaluable to me.

Table of contents

Table of contents iii
Abbreviations v
Zusammenfassung vi
Abstract vii
1 Introduction 1
1.1 Allergy 1
1.1.1 Hypersensitivity reaction type I 1
1.1.2 Hypersensitivity reaction type II 2
1.1.3 Hypersensitivity reaction type III 2
1.1.4 Hypersensitivity reaction type IV 2
1.2 Hymenoptera venom allergy 3
1.2.1 Epidemiology 3
1.2.2 Taxonomy 3
1.2.3 Hymenoptera venom 5
1.3 Clinical presentation of sting reactions 8
1.3.1 Local reactions 8
1.3.2 Systemic reactions 8
1.3.3 Unusual reaction 9
1.4 Diagnosis of hymenoptera venom allergy 10
1.4.1 Patient History: 10
1.4.2 Skin testing 10
1.4.3 Quantification of allergen specific IgE levels 12
1.4.4 Supportive tests 14
1.4.5 Issues of standard testing 14
1.4.6 Improvements of sIgE quantification 16
1.4.7 Basophil activation test 17
2 Study objectives 21
3 Material and methods 22
3.1 Patients 22
3.1.1 Inclusion criteria 22
3.1.2 Exclusion criteria 22
3.2 Testing methods 22
3.2.1 Overview 22

3.2.2 Patient history 23
3.2.3 Skin testing (Intradermal skin test) 23
3.2.4 Determination of total and specific IgE as well as specific IgG4 23
3.2.5 Tryptase determination 24
3.2.6 Basophil activation test 24
3.2.7 Sting challenge test 25
3.2.8 Clinical laboratory tests 26
3.3 Statistics 26
4 Results 27
4.1 History and demographic data 27
4.2 Sting challenge testing 27
4.3 Comparison of BAT protocols 28
4.4 Comparison of IDT, CAP, CRD and screening for CCD 29
4.4.1 Double-sensitization 29
4.4.2 Sensitization in CRD 31
4.4.3 Presence of CCD 32
4.5 Positive sting challenge 32
4.6 sIgE and sIgG4 level, ratio sIgE:sIgG4 and specific activity 34
4.6.1 Time course of sIgE levels 34
4.6.2 Time course of sIgG4 levels 40
4.6.3 Time course of the sIgE/sIgG4 ratio 46
4.6.4 Time course of the IgE - specific activity 51
5 Discussion 57
6 References 60

Abbreviations

BAT	Basophil activation test
CAP	ImmunoCAP® (sIgE determination)
CCD	Cross-reactive carbohydrate determinants
ECG	Electrocardiogram
ELISA	Enzyme-linked immunosorbent assay
FEV1	Forced expiratory volume in 1 second
FVC	Forced vital capacity
IDT	Intradermal skin test
IL-3	Interleukin 3
IUIS	International Union of Immunological Societies (system for nomenclature of allergens)
RAST	Radio allergo-sorbent test
sIgE	Specific immunoglobulin E
sIgG	Specific immunoglobulin G
SIT	Specific immunotherapy
tIgE	Total immunoglobulin E

Zusammenfassung

Insektengifte sind die häufigste Ursache einer lebensbedrohlichen Allergie. Klinisch irrelevante Doppelsensibilisierungen gegen Bienen- und Wespengift stellen ein großes Problem in der Routinediagnostik dar; sie erschweren die Auswahl der richtigen Therapie. Der Basophilen-Aktivierungstest ist ein anerkanntes ergänzendes Testverfahren in der Diagnose der Insektengiftallergie, da der Nutzen des BAT in der Insektengiftallergie in etlichen Studien gezeigt werden konnte.

Ziel dieser Studie ist es, das klinisch nicht relevante Insekt mittels BAT bei Doppelsensibilisierungen zu identifizieren. Weiters ist sie darauf ausgelegt, den negativen prädikativen Wert des Basophilen-Aktivierungstest zu erheben.

Bei 14 Patienten mit einer Anamnese einer systemischen anaphylaktischen Reaktion nach einem Insektenstich, die im Intrakutantest und in der sIgE Bestimmung eine Doppelsensibilisierung auf Bienen- und Wespengift aufwiesen, wurde ein BAT mit zwei verschiedenen Protokollen durchgeführt. Beide basieren auf der CD63- Quantifizierung von aktivierten basophilen Granulozyten. Das erste Protokoll ist als Kit kommerziell erhältlich, das zweite wurde an der Universitätsklinik für Dermatologie und Venerologie entwickelt. Negative Testergebnisse beider Methoden wurden durch Stichprovokationen mit dem jeweiligen Insekt verifiziert.

Beide Basophilen-Aktivierungstests zeigen hohe negative prädiktive Werte von 92.9% für das kommerzielle und von 87.5% für das in Graz entwickelte Protokoll. Diese Ergebnisse weisen den BAT als ein wertvolles, zusätzliches Testverfahren zur Bestimmung klinisch irrelevanter Sensibilisierung aus.

Abstract

Insect stings can cause severe, life threatening allergic reactions. Double positive results for bee and wasp venom are frequent, but often clinically irrelevant. Therefore it can be difficult to find the relevant venom for therapy. The basophil activation test is generally recognized as an additional and reliable tool in the diagnosis of hymenoptera venom allergy. Several studies have confirmed its usefulness in determining the culprit venom.

The aim of the current study was to examine its ability to identify clinically irrelevant insects and to demonstrate that a negative result in the basophil activation test has a high negative predictive value.

For that purpose 14 patients with a history of systemic anaphylactic reactions to Hymenoptera stings that showed double-sensitization in intradermal tests and sIgE quantification were analyzed with two different protocols of the basophil activation test. One protocol was commercially available as ready-to-use kit, the other was a protocol developed by the Department of Dermatology and Venerology, Graz. Negative results of the basophil activation test were confirmed by sting challenges with the respective insects.

High negative predictive values were found for both protocols. They were determined 92.9% and 87.5% for the commercially available protocol and for that developed at the Department of Dermatology, respectively. These findings clearly show that the basophil activation test is a useful additional diagnostic tool for determining clinically irrelevant sensitization.

1 Introduction

1.1 Allergy

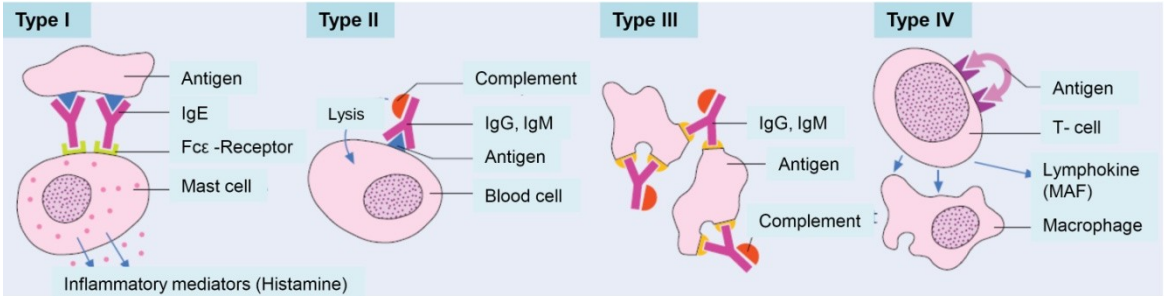
Allergy is defined as an immunologically triggered reaction to innocuous, exogenous substances (1).

Exposure to an allergen causes asymptomatic sensitization first resulting in an acquired hypersensitivity to the substance. Upon renewed exposure allergic reactions are triggered.

According to the immunological mechanisms that mediate them, hypersensitivity reactions are classified into 4 different types by Coombs and Gell. (2)

An overview can be seen in figure 1:

Figure 1: Overview over the 4 types of hypersensitivity reactions and the immunological mechanisms that mediate them. (adapted from (2))



1.1.1 Hypersensitivity reaction type I

Type-1 hypersensitivity reactions are mediated by IgE antibodies specific to allergen epitopes. IgE antibodies are predominantly found in tissues where they are produced by local plasma cells or by plasma cells of the regional lymph nodes. Via their Fc-domain they are tightly bound to high affinity receptors (FcεRI receptor) mainly on mast cells that line mucosal and epithelial tissues, but also on basophile or eosinophile granulocytes. Binding of an allergen to these bound IgE antibodies causes a cross-linking of the receptors and consequently cell activation and degranulation. Thereby preformed inflammatory mediators such as histamine, proteoglycans or tryptase as well as newly generated mediators such as leukotrienes, prostaglandins or cytokines are released.(1,3)

The thusly induced allergic reactions can be divided into two phases, the immediate and the late-phase response: The immediate response is initiated within minutes of allergen contact and lasts up to 30 minutes, until its mediators are metabolized. Its main driving factor is the mediator histamine that causes vasodilatation (erythema), increased vascular

permeability (edema), contraction of smooth muscle tissue (e.g. bronchial spasm), hyper secretion of mucosal tissue (e.g. rhinitis) and itching. In contrast, the late-phase response sets in about 6 to 12 hours after allergen exposure. Chemokines and other mediators released during and after the immediate response recruit leukocytes, such as eosinophils, macrophages or lymphocytes to the site of exposure. While late-phase reactions are medically less prominent than immediate responses, they can provoke another phase of edema and smooth muscle tissue contraction. (1,2)

Type-1 hypersensitivity reactions are triggered by soluble allergens. Examples are allergies to pollen, food, animal hair, latex or insect venom; to all of them applies, once specific IgE antibodies are produced re-exposure to the allergen triggers an allergic reaction.(1,2)

1.1.1.1 Atopy

Atopy is a predisposition towards developing IgE based hypersensitivity reactions against a variety of common environmental allergens. Atopic individuals have a higher susceptibility to allergic asthma, allergic rhinitis and atopic dermatitis; they show higher serum levels of total IgE as well as higher numbers of eosinophils in their circulation. (1,2)

1.1.2 Hypersensitivity reaction type II

Type-2 hypersensitivity reactions are mediated by IgG and IgM antibodies that bind allergens on cellular surfaces resulting in either complement induced cellular lysis or cellular destruction without complement activation. Drug-induced hemolytic anemia or thrombocytopenia and transfusion incidents belong to this type of reaction.(1,2)

1.1.3 Hypersensitivity reaction type III

Type-3 hypersensitivity reactions involve IgG and IgM antibodies directed against soluble allergens. Upon allergen contact antibody-antigen-complexes are formed that aggregate at certain tissue sites or deposit in walls of blood vessels. The consecutive activation of the complement system and leukocytes causes tissue damage. Vasculitis allergica and serum sickness are examples of type-3 hypersensitivity reactions.(1,2)

1.1.4 Hypersensitivity reaction type IV

Type-4 hypersensitivity reactions are mediated by activated T-lymphocytes that recognize the allergen as part of their epitope. In contrast to the other hypersensitivity reactions they are not an immediate response, but take 24 to 72 hours to develop. Depending on the effector cells that mediate them, different mechanisms are initiated resulting in tissue

damage. T_H1 cells predominantly activate macrophages, T_H2 cells eosinophils and cytotoxic T-lymphocytes cause tissue damage themselves. (1,2)

Type-4 hypersensitivity reactions can be further classified into three syndromes according to the way of allergen entry into the body: In contact sensitivity and gluten sensitive enteropathy allergens are absorbed through the skin or by the intestines, respectively. "In delayed-type hypersensitivity the antigen is injected into the skin" (1). Usually, allergy to insect venom elicit type 1 hypersensitivity reactions, in some cases a delayed type hypersensitivity can develop.(1,2)

1.2 Hymenoptera venom allergy

1.2.1 Epidemiology

Allergies to insect venom generally belong to IgE mediated type-1 hypersensitivity reactions. In the adult population sensitization[1] to insect venom occurs with a prevalence of 9.3 to 28.7%. Increased exposure to stings is thought to be a risk factor for sensitization. (4,5)

The incidence of insect stings in the general population is estimated between 56.6% and 94.5%; large local reactions occur with an incidence of about 19% and up to 7,5% experience systemic anaphylactic reactions after an insect sting. (5–7)

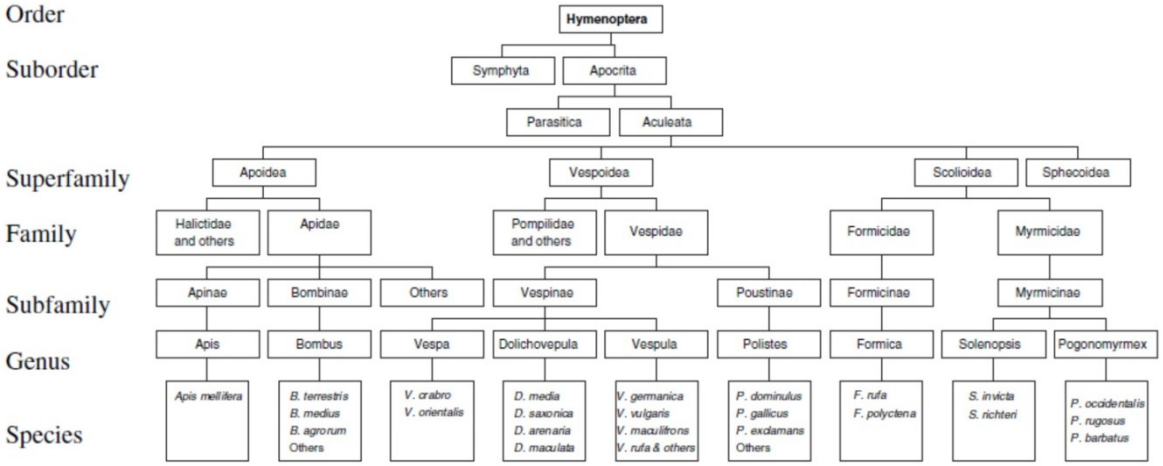
In Austria, the prevalence of Hymenoptera venom allergy in the general population was found to be 3.3%. Application of treatment is however insufficient. Only 15.2% of patients with hymenoptera venom allergy receive specific immunotherapy, while 41.3% go without treatment. (8)

1.2.2 Taxonomy

Insects responsible for allergic reactions predominantly belong to the order of Hymenoptera. (9–11)

[1] Sensitization: "indicated by a positive skin test and/or the detection of specific IgE in patients with no previous case history"(4)

Figure 2: Taxonomy of Hymenoptera (taken from(9))



Aculeata is a suborder of Hymenoptera. It comprises social insects that have established a class of sterile female workers responsible for nest-building, brood care and defense. Their ovipositor developed into a stinging apparatus that is no longer capable of laying eggs but used to deliver venom.(9)

Most allergic reactions to insect venom are triggered by representatives of three families, Apidae (bees), Vespidae (wasps) and Formicidae (ants): The genera Apis (bees) and Bombus (Bumble-bees) are part of the family Apidae, whereas the family Vespidae comprises the subfamilies Polistinae and Vespinae, with Vespa (hornets), Vespula (wasps) and Dolichovespula as significant genera. Of the family Formicidae the genera Solenopsis and Pogonomyrmex are medically relevant. (9,12)

Figure 3: Apis mellifera (taken from (13))

Figure 4: Vespula germanica (taken from (13))

Regarding the different species there are differences in the geographic distribution and therefore in their allergenic relevance: In central and northern Europe mainly honeybees

belonging to the species Apis mellifera (referred to in the following as bees) and different species of wasps, such as Vespula vulgaris or Vespula germanica (referred to in the following as wasps) elicit anaphylactic reactions to their venom; stings of bumble-bees and hornets are rarely seen. (9,11,12) In Mediterranean countries wasps and some species of Polistinae are of importance. In Europe also some rare cases of allergic reactions to ants of the species F.rufa have been described.(9)

As this study is conducted in Austria its focus is put onto the medically most relevant Hymenoptera species, bees and wasps.

1.2.3 Hymenoptera venom

The venom of bees and wasps is a conglomerate of many different substances that include several pharmacologically active compounds. They can be classified into low-molecular weight compounds, peptides and proteins that often have enzymatic properties.(10,11)

An overview is given in table 1.

Table 1: Composition of bee and wasp venom

	Bee venom	Wasp venom
Low-molecular weight compounds 20-25%		
Biogenic Amines:		
– Histamine, Catecholamines	+	+
– Acetylcholine, Serotonin	-	+
– Amino acids, Oligopeptides	++	++
– Carbohydrates, Phospholipids	+	+
– others		
Peptides 50-60%		
	Melittin (Api m 4) Api m 6 (Api m 6) Apamin Mast cell degranulating peptide others	Kinins Mastoparan Chemotactic peptides others
Proteins 15-30%		
	Phospholipase A_2 * (Api m 1) Hyaluronidase * (Api m 2) Acid phosphatase * (Api m 3) Dipeptidylpeptidase IV -homologue (Api m 5) Serine protease (Api m 7) Carboxylesterase (Api m 8) Serine-carboxyl-	Phospholipase A_1 * (Ves v 1) Hyaluronidase * (Ves v 2) Dipeptidylpeptidase IV-homologue (Ves v 3) Protease (Ves v 4) Antigen 5 * (Ves v 5) Phosphatase

	peptidase	(Api m 9)
	Icarapin	(Api m10)

* major allergens

Api m x and Ves v x represent the IUIS-nomenclature of the different bee and wasp allergens, respectively. (adapted from (10), (9,13–17))

Peptides in combination with phospholipases are the main cause of local toxicity and the induction of pain. (10,11) In bee venom these peptides include the neurotoxin apamin, a mast cell degranulating peptide (13) and melittin that, besides being an allergen, acts synergistically with phospholipases in causing cellular lysis (18). Wasp venom contains chemotactic peptides that play a role in recruiting leukocytes to the site of stinging, mastoparan that induces degranulation in mast cells (13) and wasp kinins that together with serotonin and acetylcholine are considered to be responsible for local pain (19).

In addition, low-molecular weight compounds, mostly biogenic amines such as histamine act via their vasoactive properties as "spreading factors"(11), which is further enhanced by hyaluronidases. (10,11)

The known allergens of bee and wasp venom are proteins, mostly glycoproteins, of "10-50 kDa containing 100-400 amino-acid residues"(9). The two exceptions are the peptides melittin and Api m 6 with molecular weights of 2.9 kDa (26 amino acid residues) and 7.9 kDa (35 amino acid residues), respectively. Allergens are classified according to their allergic potential into a major and a minor group, where major allergens elicit the formation of specific IgE antibodies in more than 50% of the allergic individuals. (9–12,20)

An overview over the known allergens in bee and wasp venom can be found in table 1.

1.2.3.1 Bee venom allergens

The major allergens in bee venom are phospholipase A_2 (Api m 1), hyaluronidase (Api m 2) and acid phosphatase (Api m 3) (21), where Phospholipase A_2 is considered to be the most potent allergen. It is a glycoprotein that makes up 12-15% of the bee venom's dry weight. There are several minor allergens in bee venom, such as melittin (Api m 4), Api m 6 (20), and icarapin (22) (table 1). While melittin is the major component of bee venom, only 28% of patients develop specific IgE antibodies against it. (9–11,17,18,20)

1.2.3.2 Wasp venom allergens

There are 5 known components of wasp venom that can trigger allergic reactions in patients. The major allergens are phospholipase A_1 (Ves v 1), and antigen 5 (Ves v 5),

where Ves v 5 is considered to have the highest allergic potential. Hyaluronidase (Ves v 2) is one of three minor allergens in wasp venom. It has formerly been considered to be a major allergen, however sIgE were found to react mainly against its carbohydrate side chains while only 10 to 15% of patients show sIgE against its protein backbone. (9–11,13,17,23,24)

1.2.3.3 Sequence homologies of allergens

All known allergens in bee and wasp venom are proteins. They can show amino-acid sequence homologies to allergens of other Hymenoptera species. Among the different wasp species allergens display sequence identities up to 95%. They also show about 67% sequence homologies to allergens of hornet (vespa) venom causing IgE cross reactivity between wasp and hornet allergies. For bee venom, the main allergen phospholipase A_2 (Api m 1) displays 53% sequence identity to phospholipase A_2 in bumble-bee venom resulting in IgE cross reactivity between bee and bumble-bee allergies. (9,12)

Among allergens in bee and wasp venom the phospholipase allergens Api m 1 and Ves v 1 show no sequence homologies. Cross reactivity can occur on the basis of two allergen groups, hyaluronidases (Api m 2 and Ves v 2) and dipeptidylpeptidases (Api m 5 and Ves v 3), where the Api m 2 and Ves v 2 display 50% sequence identity making hyaluronidases a major cross-reactive component. (9,12,14)

1.2.3.4 Venom dosage per sting

As the stinging apparatus developed from the ovipositor, only female bees and wasps are capable of stinging. The amount of venom that is delivered with a single sting varies. The stinger of bees is barbed. While it can be retracted from other insects, the stinger with the attached venom sac remains in human skin, where it continues to release venom for about one minute. The bee is therefore only able to sting humans once and dies shortly afterwards. With one sting 50 to 140 µg of venom are injected. (9,11)

In contrast, the stinger of wasps has no barbs and can be retracted from human skin thereby enabling wasps to sting multiple times without dying. A wasp sting delivers between 1.7 to 3.1 µg of venom. (9,11)

1.3 Clinical presentation of sting reactions

Bee or wasp venom delivered with a sting can trigger three different types of reactions: local, systemic or unusual reactions. (5,9,11)

1.3.1 Local reactions

Local reactions to hymenoptera stings can further be classified into normal local reactions and large local reactions, where the later shows a prevalence ranging from 2.4 up to 38% in the general population (4).

1.3.1.1 Normal local reactions

Normal local reactions represent the response of non-allergenic individuals to hymenoptera stings comprising pain, erythema and slight swelling of less than 10 cm in diameter at the stinging site. While these symptoms subside within 24 hours, a small local reaction can persist for few days. (9,13)

1.3.1.2 Large local reactions

In contrast, large local reactions are characterized by a swelling at the site of stinging larger than 10 cm of diameter that persists longer than 24 hours. They also include pain and erythema. In addition, unspecific inflammatory symptoms, such as “a feeling of sickness, shivering, fever or headaches” (9) as well as a swelling of local lymph nodes can occur. The mechanisms underlying large local reactions are unknown and could be IgE- or cell-mediated, or both. Depending on the location of the sting large local reactions can be life-threatening (e.g. after stings in the upper airways). (9,11)

1.3.2 Systemic reactions

For systemic reactions symptoms manifest beyond the site of stinging. The most severe response to hymenoptera stings are systemic anaphylactic reactions. (9,11)

1.3.2.1 Anaphylaxis

Anaphylaxis is an acute serious allergic reaction that involves different organ systems of the body and can lead to death. Anaphylactic reactions are predominantly IgE mediated. They are generally triggered by a single sting; symptoms manifest immediately upon exposure to the allergen, usually within minutes or even seconds, but in rare cases after hours or days. Organ systems that can be affected are the skin, the gastrointestinal tract, the cardiovascular, respiratory and central nervous system. Which of these are involved varies

not only among patients but also between different episodes. Clinical experience showed that a fast onset of symptoms usually correlates with severity of the symptoms. (2,3,9,11,25) Anaphylaxis can be fatal, caused by bronchial obstruction, cardiovascular failure or disseminated intravascular coagulation. In most cases though, recovery from an anaphylactic reaction occurs without any complications. However it is a traumatic experience that can lead to behavioral changes to avoid a re-sting, thereby impairing the quality of life on emotional, social and sometimes professional levels. (9,11)

"Diagnosis of anaphylaxis is primarily based on clinical criteria" (3). Standard diagnostic tools, such as skin testing or IgE quantification are only able to confirm sensitization to allergens. Anaphylactic reactions are classified according to their severity into four categories by J. Ring and Messmer. (3,9,25) An overview is given in table 2.

Table 2: Classification of systemic reactions modified according to J. Ring and Messmer

Grading	Clinical symptoms
Grade I	Generalized skin symptoms (e.g. flush, generalized urticaria, angioedema)
Grade II	Mild to moderate pulmonary, cardiovascular, and/or gastrointestinal symptoms
Grade III	Anaphylactic shock, loss of consciousness
Grade IV	Cardiac arrest, apnoea

The grade of reaction is determined by the most severe symptom, where none of the symptoms are mandatory (25). (taken from (9))

1.3.2.2 Systemic toxic reactions

When delivered in higher doses, hymenoptera venom can cause serious symptoms, such as rhabdomyolysis, hemolysis, acute renal failure, hepatic dysfunction or coagulation disorders, that can even be fatal. Systemic toxic reactions require multiple stings, usually 50 to several hundred, and occur rarely. (9,11)

1.3.3 Unusual reaction

Reactions to hymenoptera stings may manifest in unusual ways, showing for instance serum sickness like symptoms, lymphadenopathy, neurological, renal or cardiovascular disorders. The mechanisms underlying unusual reactions are unknown. Such cases are however extremely rare and their correlation to occurred hymenoptera stings is often doubtful. (9,11)

1.4 Diagnosis of hymenoptera venom allergy

Hymenoptera venom allergy is a serious health problem. Specific immunotherapy with the responsible hymenoptera venom is considered the only effective, long-lasting treatment and can successfully prevent systemic reactions to further stings in 75-95% of patients (11). For the choice of venom administered in immunotherapy identifying the culprit insect is paramount. Current diagnostic tools comprise patient history, skin testing and quantification of sIgE antibodies. These procedures, however, are not sufficient in all cases. Both skin testing and sIgE quantification often show double positive results to bee and wasp venoms simulating double sensitization, which poses difficulties in finding the right venom for treatment. The basophil activation test (BAT) is an additional, reliable diagnostic tool that is able to demonstrate functional responses and thus reduces the frequency of clinically irrelevant double-sensitizations. (6,11,26,27)

1.4.1 Patient History:

The patient history should include all previously occurred sting reactions. The date of the reaction, the stinging site, the symptoms and severity of the reaction, the interval between the sting and manifestation of the symptoms, the acute treatment, the risk factors for anaphylaxis, the risk for repeated stinging incidents, and other allergies need to be covered. For most patients it can be difficult to differentiate between bees and wasps. Additional questioning can give clues to determine the culprit insect. (9,11)

Table 3: Clues that can help to determine the culprit insect (taken from (11))

Bee	Wasp
Rather more “peaceful” (except near beehives)	Rather more “aggressive”
Spring to late summer (also on warm winter days)	Summer to late autumn
Stinger remains in the skin after stinging	Usually retract stinger after stinging
Mainly near beehive or flowers	Usually around food or waste

1.4.2 Skin testing

Skin testing is used to determine sensitization to different types of hymenoptera venom and involves the introduction of purified venom extracts into the skin of patients. As this can cause systemic reactions to the venom, testing should occur with incremental venom concentrations. The more sensitive a patient is to the allergen, the lower the allergen

concentration required to elicit a response. However, positive skin tests are not able to predict occurrence or severity of anaphylactic reactions after allergen-exposure. For skin testing there are two different procedures that can be performed: Either skin prick testing followed by intradermal testing in case of inconclusive test results, or strictly intradermal testing. The ability of mast cells in the skin to react to re-exposure shortly after allergen contact is limited. To avoid false negative test results, skin testing should be carried out at least two weeks after a stinging reaction. (2,3,9,11,28–30)

1.4.2.1 Skin prick test

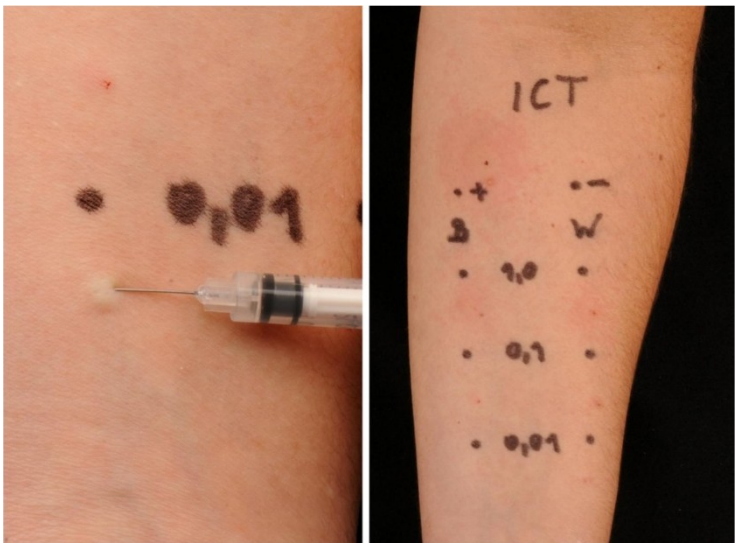
For the skin prick test different hymenoptera venom extracts are introduced into the patient's skin using lancets. Usually, venom concentration ranging from 10 to 300 µg/ml are used. 15 to 20 minutes after introduction the test result is examined, where a wheal with a diameter 3 mm larger than the negative control reaction is considered a positive test result and indicates the presence of mast-cell bound sIgE. (2,9,31)

1.4.2.2 Intradermal (intracutaneous) test

For the intradermal test (IDT) different hymenoptera venom extracts are administered intracutaneously into the skin of the volar surface of the forearm. Usually, 0.02 ml of venom at concentrations between 0.001 and 1 µg/ml are used. 15 to 20 minutes after injection the test results are examined, where a wheal of 5 mm in diameter accompanied by surrounding erythema is considered a positive test result. (2,9)

The sensitivity of the IDT is considered much higher than that of the skin prick test; it is estimated at 90% or higher for venom concentrations of 1 µg/ml. The specificity of skin tests is difficult to define, because sensitization can occur with any further stings. It was estimated between 80 and 90% (table 4) (9,10)

Figure 5: Intradermal skin testing



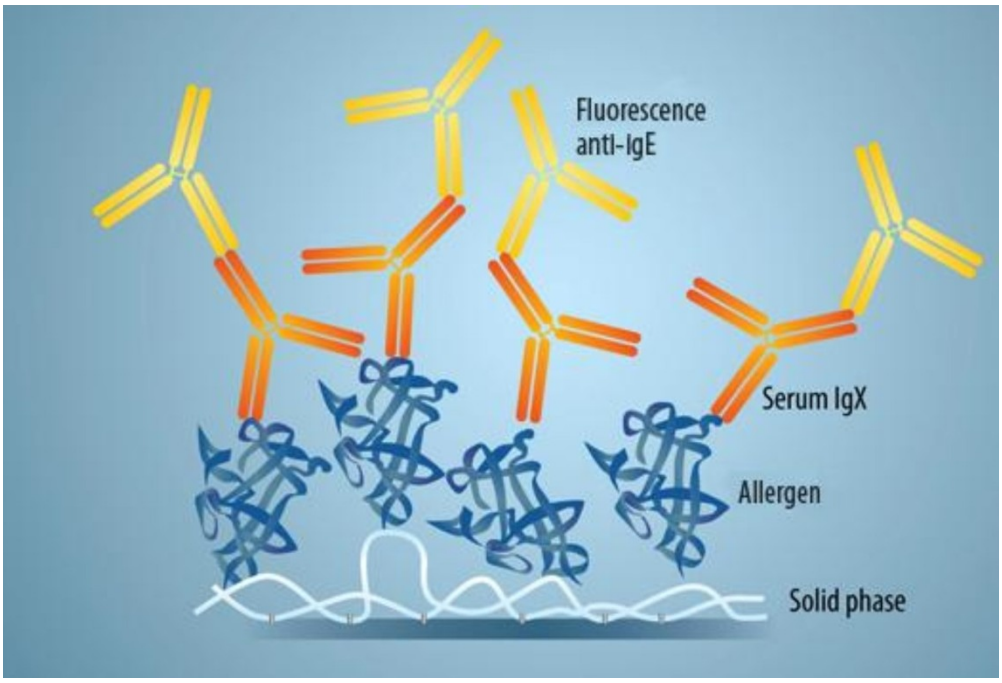
Intradermal application of allergen extracts (A) and positive test results showing wheals with surrounding erythema (B); (kindly provided by Gunter Sturm)

1.4.3 Quantification of allergen specific IgE levels

Allergen specific IgE (sIgE) antibodies are the mediators of type-I-hypersensitivity reactions. Measuring their levels in patients' sera is therefore a valuable diagnostic tool to determine sensitization to different allergens. There is however no clear correlation between sIgE levels and a patient's response to allergen exposure. High sIgE serum-levels do not predict the occurrence and severity of anaphylactic reactions after a sting or during immunotherapy. (9,29,30)

Methods used to measure sIgE serum-levels are in vitro techniques derived from the Radio Allergo Sorbent Test (RAST). Present-day sIgE assays are ELISA techniques such as the ImmunoCAP®ISAC (Phadia) that use biochip technologies: Allergens are immobilized on a solid phase. Human serum or plasma is added causing allergen-specific antibodies of all isotypes to bind to the immobilized allergens. After a washing step to remove unbound proteins, fluorescence- labeled anti-human IgE Fc antibodies are added to detect bound IgE. Following another washing step to remove unbound detection antibodies, test results are measured with a laser scanner. As this assay is a semi-quantitative test, results are reported in Standardized Units. (figure 6) (32–34)

Figure 6: Test principle of the ImmunoCAP® ISAC



Allergens are immobilized on a solid phase. Specific antibodies of all isotypes from patient serum or plasma bind to the immobilized allergens. Allergen-bound IgE are detected with fluorescence-labeled anti IgE Fc antibodies (33). (taken from (35))

sIgE should be determined within the first year but at least four weeks after a stinging incident, as the sIgE serum-levels are highest during this period. (9,10) The sensitivities of sIgE assays are found to be lower than that of IDT, ranging from 76 to 91%. Determining the specificity is similarly challenging as for the skin testing; it was estimated at 66.7 to 85%. (30,36,37). (table 4)

Table 4: Sensitivity and Specificity of intradermal skin test and sIgE quantification (9,10,30,36,37)

	Sensitivity	**Specificity**
Intradermal test	90-100%	80-90%
sIgE quantification	76-91%	66.7-85%

1.4.3.1 IgE-specific activity

The specific activity is the ratio of sIgE:tIgE and was suggested to be a marker for IgE antibody responses. The higher the specific activity of a certain sIgE, the higher is the probability of a high surface density of this sIgE on mast cells and basophils. It is thought that this can give an estimation of cellular activation and the magnitude of mediator release upon allergen encounter. (38)

1.4.4 Supportive tests

1.4.4.1 Detection of allergen-specific IgG

Allergen-specific IgG (sIgG) serum levels reflect the extent of exposure to allergens. After allergen contact, due to an insect sting or SIT, sIgG levels increase and remain elevated throughout weeks and even month. Afterwards they decrease more rapidly than sIgE levels. Thus, sIgG quantification can help to identify the culprit insect and is a valuable tool for monitoring and adjusting effective immunotherapy. Clinically successful SIT generally shows high levels of sIgG, in particular sIgG4. sIgG4 is thus thought a "blocking antibody" involved in providing some measure of protection. (29,39) However, the clinical use of sIgG is limited to SIT. sIgG levels do not correlate with the occurrence of systemic reactions and cannot be used to predict them in cases of re-exposure. Concerning quantification techniques, measuring sIgG levels is performed similar to sIgE quantification with different RAST-based assays. (9,10,29,32)

1.4.4.2 Baseline serum tryptase

Tryptase is an enzyme that is almost exclusively produced by tissue mast cells and released upon cellular activation. Basophils express tryptase only in low levels, 300- to 700-fold less than mast cells. For that reason serum tryptase levels are considered a marker to estimate mast cell number and activation. Healthy individuals show baseline serum tryptase levels up to 11.4 µg/L, while levels above 20 µg/L are associated with mastocytosis. Elevated serum tryptase and mastocytosis are considered a risk factor increasing the severity of anaphylaxis. In patients with a history of systemic sting reactions grade two and above determination of baseline serum tryptase levels is recommended. (10,17,32,40)

1.4.5 Issues of standard testing

The standard diagnostic tools of hymenoptera venom allergy comprise patient history, skin testing and quantification of sIgE. However, it is estimated that in up to 59% of patients suffering from hymenoptera venom allergies these tests show positive results for both, bee and wasp venom mimicking double-sensitization (41). Thereby difficulties for the selection of venom for SIT arise. (42)

Concerning the quantification of sIgE, 30-40% of patients' sera show in-vitro reactivity with components of both types of venom (17,43). This double positivity can be due to four possible causes: true double sensitization, cross-reactivity to either protein- or carbohydrate

based epitopes or non-specific IgE absorption: (43) True double-sensitization means independent sensitization to different allergens of bee and wasp venom. In that case systemic allergic reactions may be triggered by stings of both insects and therefore treatment with both types of venom is required. True double-sensitization is however rare and double-positivity is largely attributed to molecular cross-reactivity, where treatment with one type of venom, that of the primarily responsible insect, is sufficient. (42,43)

IgE cross-reactivity can be based on protein or carbohydrate epitopes: Protein based cross-reactivity can occur where allergens of bee and wasp venom display homologies in their amino-acid sequences, as for hyaluronidases or dipeptidylpeptidases (see section 1.2.3.3.). (14,44) Up to 80% of IgE cross reactivity is based on cross-reactive carbohydrate determinants (CCD) (15,26), carbohydrate structures found on glycoproteins of plants and insects. While CCD-sIgE antibodies appear clinically irrelevant in most patients, they represent a main source of interference for in-vitro sIgE quantification. (15,43). (see section 1.4.6.1.)

In-vitro cross-reactivity can also be caused by "non-specific absorption of IgE to the allergosorbent" (43), a phenomenon that appears more frequently at very high tIgE serum levels and is associated with binding of low-affinity sIgEs: Affinity of antibodies to their antigen correlates to the stability of the antibody-antigen-complex, where low affinity leads to a faster dissociation of that complex. In-vivo, the trigger of mast-cell and basophil activation is cross-linking of at least two FcεRI receptors, which occurs by stable binding of antigens to their attached IgE antibodies. Thus, high-affinity antibodies are required to activate these effector cells. Concerning allergic reactions, low-affinity antibodies are considered less relevant; they may cause basophil activation in the presence of high-affinity antibodies. However, the in-vitro situation does not reflect the situation in vivo: In vitro sIgE of different affinities, including low-affinity antibodies, bind to the allergens of the allergosorbent. Moreover binding is monovalent and since every bound sIgE will generate a signal in the assay, cross-linking is not required. (43,45,46)

In addition, negative sIgE serum-levels don't necessarily exclude allergy, particularly in patients with low tIgE, since sIgEs bound to the cellular surface of effector-cells are not covered by serological assays. (10)

Concerning IDT, CCD as well as low-affinity antibodies are considered to play no role in eliciting a response. Nevertheless, positive rest results of questionable clinical relevance are observed frequently. (45)

1.4.6 Improvements of sIgE quantification

1.4.6.1 Screening for cross-reactive carbohydrate determinants

CCD are pan-allergens, they are widely distributed on glycoproteins of plants and lower animals, such as hymenoptera, but not on that of mammals. The core structures of cross-reactivity are alpha 1,3 fucosylated N-glycans that serve as IgE epitopes. Sensitization to CCD develops through contact mainly with pollen and plant food products but also through insect stings. (17,26) While CCD-sIgE antibodies appear clinically irrelevant in most patients, they represent a main source of interference for in-vitro sIgE quantification. (15,43). Many hymenoptera venom allergens are glycoproteins containing CCD. CCDs are more abundant in bee venon allergens, where almost all allergens are glycosylated. Wasp venom includes only some glycosylated allergens; the major allergens Ves v 1 and 5 are even CCD-free. Table 5 gives an overview over the major allergens of bee and wasp venom and their extent of glycosylation. Hyaluronidases (Api m 2 and Ves v 2) are heavily glycosylated. Cross-reactivity between Api m 2 and Ves v 2 is largely attributed to CCD, while cross-reactivity through their amino-acid backbones is rare. When neglecting IgE binding to CCD, the relevance of hyaluronidases as allergens becomes minor. (14,24) Nevertheless, Api m 2 has shown to be relevant for bee venom allergies also beyond its carbohydrate structures (47). In contrast, the allergens Ves v 5 and Ves v 1 are not glycosylated, which makes them a useful target for component resolved diagnosis (CRD) (44).

Table 5: Glycosylation of major allergens of bee and wasp venoms, where (x) shows the number of potential glycosylation sites (adapted from (15), (17,44,45))

	Allergen	IUIS-nomenclature	Glycosylation
Bee venom	Phospholipase A_2	Api m 1	Glycosylated (1)
	Hyaluronidase	Api m 2	Glycosylated (4)
	Acid Phosphatase	Api m 3	Glycosylated (4)
Wasp venom	Phospholipase A_1	Ves v 1	Non- glycosylated
	Antigen 5	Ves v 5	Non- glycosylated

Screening for CCD-sIgE is performed with allergens containing a high portion of CCD. For that purpose glycoprotein-containing extracts and single glycoproteins, such as natural rubber latex extracts, horseradish peroxidase, ascorbic acid oxidase, bromelain or its isolated N-glycan, MUXF, can be used. However, the presence of CCD-sIgE does not

exclude the possibility of protein-based cross-reactivity, neither that of true double-sensitization. (17,26,45)

1.4.6.2 Component resolved diagnosis

In general, skin testing and sIgE quantification are performed with whole venom extracts, thereby identifying the allergen source, but not the molecular entities eliciting the reaction.
In component resolved diagnosis (CRD) sIgE against individual allergens are determined in an approach to identify a patient's sensitization profile. (13) For that purpose different recombinant allergens of bee and wasp venom are used. Depending on the allergens and the expression systems that were used, they are available in glycosylated and non-glycosylated forms. Since glycosylated allergens are prone to CCD reactivity, it was suggested that non-glycosylated recombinant allergens may be better suited for CRD. (44,48)
CRD can help to identify the culprit insect and to distinguish true double-sensitization from cross-reactivity. Currently, only few allergens are commercially available: Api m 1, Ves v 1 and Ves v 5. rApi m 1 has emerged as additional marker for bee venom allergy, determination of sIgE against it show sensitivities ranging from 62 to 79%. For wasp venom allergies, sIgE quantification for rVes v 5 shows a sensitivity of about 88%, which can be improved to up to 96% when combined with detection of sIgE against rVes v 1. (14,44)

These additional tests help improve diagnosis of hymenoptera allergy and can provide useful information for identifying the culprit insect in some cases. Nevertheless they are not sufficient in all of them. Double-positive test results of questionable clinical relevance are still observed frequently. In addition, sensitivity of Api m 1 can be very low.
In cases where standard diagnostics show contradictory or equivocal results, cellular tests can be used to demonstrate sensitization. (27,49) The basophil activation test is a cellular testing system that is generally recognized as an additional and reliable diagnostic tool. Compared to standard tests it has the advantage of being an in-vitro assay able to demonstrate functional responses. (6,50)

1.4.7 Basophil activation test

Cellular activation in basophil granulocytes causes not only the release of inflammatory mediators, but also an up-regulated expression of cellular surface markers, in particular

CD63 and CD203c. The basophil activation test (BAT) is a cellular, in-vitro assay based on flow-cytometric quantification of one of these markers after allergen exposure (51)

1.4.7.1 CD63 versus CD203c

There are two different approaches of quantifying basophil activation. They differ in the cellular markers they utilize; either CD63 or CD203c. In the 1990s a protocol measuring CD63 up-regulation in activated basophils was described by Sainte-Laudy and Sabbah. 2001 a CD203c-based assay was developed. Major differences of these molecules as basophil activation markers are shown in table 6. (17,50,52)

Table 6: Differences in regulation of CD63 and CD203c expression (adapted from (52), (50))

	CD63	**CD203c**
Synonym	Gp43:lysozyme-associated membrane protein	E-NNP3* enzyme
On resting basophils	Barely detectable	Constitutively present
Present on up-regulation	– Other blood cells, platelets – Maximum within 25 to 30 min – Associated with mediator release	– Basophil specific – Maximum within 10 to 20 min – Not associated with mediator release
Induction by IL-3*	No	Yes
Induction by PGD2*	No	Yes
clinical diagnostic efficiency	No clear agreement in literature	
Clinical validation	CD63 >> CD203c	

*Abbreviations: E-NNP3: ectonucleotide pyrophosphatase/phosphodiesterase 3
IL-3: Interleukin 3
PGD2: Prostaglandin D2

While CD63 is not only expressed in basophils, but also in other blood cells and platelets upon their activation, CD203c is rather basophil specific. It is found in blood basophils, tissue mast cells and their progenitors, but not in other blood leukocytes. However, CD203c is constitutively present on plasma-membranes of resting basophils, whereas CD63 is barely detectible on them. Another difference lies in the expression kinetics of the two markers. Where CD203c up-regulation is influenced by Prostaglandin D2 and IL-3, that of CD63 is closely related to basophil degranulation. (50,52) In resting basophils CD63 is expressed on the insides of vesicle membranes. During degranulation in activated basophils, vesicles fuse to the plasma membrane and CD63 is consequently transferred to the cellular surface, where it can be detected by flow-cytometry. (30,53)

The main advantage of CD63 as marker for basophil activation though is that it is clinically validated and thus generally recognized as an additional and reliable diagnostic tool. (52)

1.4.7.2 The BAT technique

Either whole blood (heparinized or EDTA blood) or isolated leukocytes are incubated with allergen at different concentrations. IL-3 may be added as stimulant. Depending on the utilized marker for basophil activation, CD63 or CD203c, and the mode of identifying basophils within the cellular mixture, staining occurs with different, fluorescent labeled antibodies. There are several strategies of "capturing" basophils which include polyclonal anti-IgE antibodies, a combination of anti-IgE and anti-CD203c antibodies or of anti-CD123 (IL-3 receptor) and anti-HLA-DR (used to exclude monocytes and antigen-presenting cells) antibodies. The most practical and reliable approach is the labeling with CCR3 (54). The up-regulated expression of activation marker is quantified by flow-cytometry. (32,52)

1.4.7.3 Advantages of BAT

In contrast to standard tests, the BAT is a cellular in-vitro assay that has the advantage of showing functional responses: Also skin tests are able to demonstrate functional responses, but as an in-vivo technique the danger of systemic reactions to administered insect venom remains. In addition, the BAT can be performed under anti-histamine (H_1-blockers) treatment. While H_1-blockers interfere with skin tests, they do not affect mediator release from basophils, nor the consequent up-regulation in CD63 expression. (6)

Specific IgE quantification is the standard in-vitro assay for the diagnosis of Hymenoptera venom allergy. However, the BAT reflects the in-vivo situation more accurately, where only cross-linking of antibodies and not monovalent binding, as for sIgE quantification, yield a positive response. (6)

Several studies have demonstrated the usefulness of the CD63-based BAT as additional diagnostic tool in Hymenoptera venom allergy (30,37,49). Its sensitivity was found comparable to that of skin and sIgE testing, ranging from 89% to 100%. (17,30,37,55) Pre-incubation with stimulating IL-3 was suggested to increase sensitivity (55). Specificity of the CD63-based BAT has shown to exceed that of sIgE determination, it was reported between 86.7 and 100%. (30,51,55). In addition, double-sensitization, a major issue of standard tests, is less frequently observed in the BAT than in CAP (17,1% vs. 61,5%) (45).(30)

1.4.7.4 Issues of BAT

Despite the various advantages of the BAT, there are few challenges that need to be addressed: As for IDT and sIgE quantification, there is no correlation between the extent of basophil activation and the severity of clinical symptoms upon allergen exposure. (30) Moreover, in the in-vitro situation CCDs sometimes can stimulate even basophils, thereby leading to positive results of questionable clinical relevance. (45)

There are also some technical challenges. Blood storage conditions are one of them. Medium, storage time and storage temperature can affect the extent of reactive basophils within a sample. (52) For ideal cellular performance, blood samples should be analyzed as quickly as possible, preferably within 4 hours after blood taking. A reduction of basophil reactivity in EDTA blood by up to 58,5% has been observed for storage periods exceeding 48 hours at 4°C (6). Even for shorter storage periods increased false negative results may occur. The dispatch of samples over hours or days to specialized laboratories is thus often not recommendable. (6,17) Another challenge is comparability of results gained by different BAT testing systems. Several factors including storage conditions, staining and the used flow-cytometer systems may influence quantitative outcomes. In addition, various in-house protocols are often not standardized and may yield different results. Comparability of BAT results is thus only given if identical settings are applied. (6)

2 Study objectives

The main objective of this study was to evaluate the ability of the BAT to determine the clinically irrelevant insect venom and to demonstrate that a negative result in the BAT has a high negative predictive value.

3 Material and methods

3.1 Patients

Patients with insect venom allergies were selected on the basis of the following inclusion and exclusion criteria.

3.1.1 Inclusion criteria

Legally competent women and men between 18 to 65 years of age with a history of one or more anaphylactic reactions (≥ grade II according to the classification by J. Ring and Messmer; see table 2) to bee or wasp stings and double sensitization to bee and wasp venom in IDT and CAP.

3.1.2 Exclusion criteria

- Individuals who have received immunotherapy with the BAT-negative insect venom
- Individuals with severe chronic illness
- Severe asthma (FEV1 < 80% of predicted, FEV1/FVC ratio < 70%)
- Severe disorder of the lungs, liver , kidneys or nervous system
- Clear chronic or acute cardiovascular failure
- Poorly controlled hypertension and/or severe chronic ischemic heart disease
- Patients of ACE-inhibitor or beta-blocker treatment
- Severe psychological disorders
- For females: pregnancy and breast-feeding

3.2 Testing methods

3.2.1 Overview

Table 7 gives an overview over the different procedures that were carried out during the course of the study. Inclusion of patients occurred after performing the screening procedures.

Table 7: Overview over the different study procedures performed during each phase of the study

Study Procedures	Screening	Testing	Monitoring
Patient history	X		
Intradermal skin testing	X		

tIgE/sIgE/sIgG4 determination	X		X
Baseline serum tryptase	X		
Basophil activation test		X	
Sting challenge test		X	
Clinical laboratory	X		
Vital signs*, ECG**	X	X	
Spirometry	X	X	

*Vital signs: Systolic and diastolic blood pressure, pulse, arterial oxygen saturation

**12-lead electrocardiogram (ECG)

3.2.2 Patient history

Previous hymenoptera stings, the course of reactions to the stings, the medical and allergic history and the current medication were recorded.

3.2.3 Skin testing (Intradermal skin test)

Pre-screening of patients occurred by IDT using standardized endpoint titration (0.02 ml of 0.01; 0.1; and 1 µg/ml solutions) with purified bee and wasp venom extracts (ALK-Abello, Denmark). As positive control histamine 0.01% and as negative control saline 0.9% were used. 15 minutes after intradermal injection the test was evaluated. A wheal of ≥5mm in diameter accompanied by surrounding erythema was considered a positive reaction.

Patients showing double sensitization to bee and wasp venom in IDT were considered to be included in the study.

3.2.4 Determination of total and specific IgE as well as specific IgG4

During the screening process a venous blood sample of about 24 ml was taken from patients. tIgE, sIgE and sIgG4 against bee and wasp venom, rApi m 1, rVes v 5, rVes v 1, MUXF3-Bromelain (CCD) as well as sIgE against timothy grass, birch, house dust mite, cat, dander and cow's milk protein for atopy screening were determined.

During the monitoring phase, 3 hours, 7±1 days and 30±5 days after the sting challenge about 8ml of venous blood were taken for a reassessment of sIgE and sIgG4 levels against bee and was venom, rApi m 1, rVes v 5, rVes v 1 and MUXF3-Bromelain.

Determination of tIgE, sIgE and sIgG4 were performed by ImmunoCAP® (Phadia, Sweden) according to the manufacturer's instructions. Relative sIgE concentrations were expressed as percentage of tIgE.

3.2.5 Tryptase determination

Baseline serum tryptase levels were determined to exclude mastocytosis as cause of non-specific sting reactions. The tryptase levels were measured by ImmunoCAP (Phadia, Sweden) according to the manufacturer's instructions. Levels >11.4µg/L (95^{th} percentile of the general population) were considered to be elevated.

3.2.6 Basophil activation test

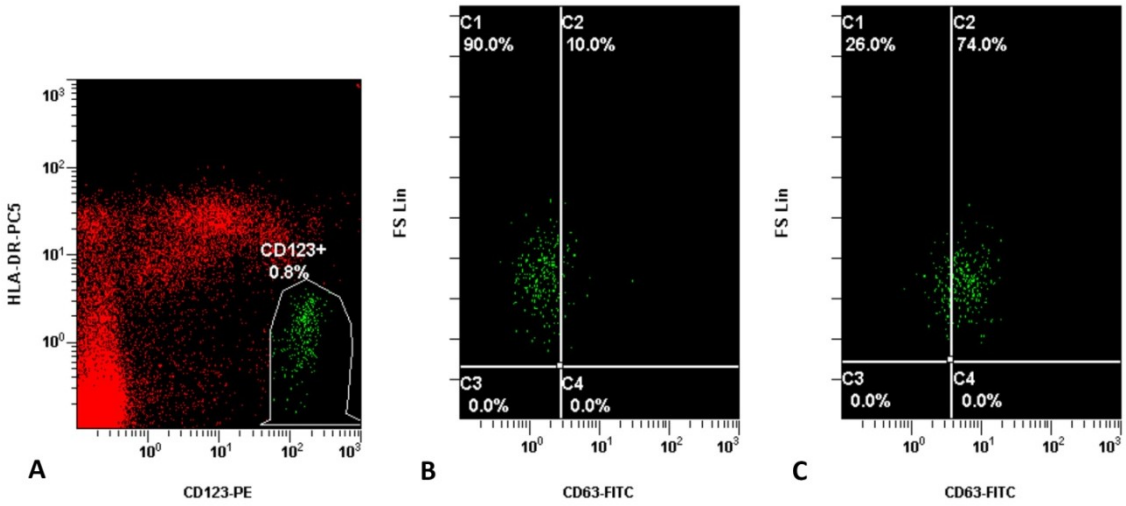
BAT was performed with patients that showed double sensitization to bee and wasp venom in IDT and CAP using two different BAT protocols. For that reason, a venous blood sample of 12ml was taken.

For the first protocol 2 ml of EDTA whole blood were stained with anti-CD63 FITC-conjugated antibody (Beckman Coulter, Austria), anti-HLA-DR PC5-conjugated antibody (Beckman Coulter, Austria) and anti-CD123 PE-conjugated antibody (BD Biosciences, Austria) at a ratio of 1:50 as well as Interleukin 3 (SIGMA, Austria) at a ratio of 1:340 and incubated at room temperature for 10 minutes. To determine CD63 up-regulation in response to allergens, four serial dilutions (200, 20, 2 and 0.2 E/ml) of each, bee and was venom (ALK-Abello, Denmark) with PBS^+ buffer were prepared. As positive control four serial dilutions of monoclonal-anti-IgE antibody (2 µg/ml, 0.4 µg/ml, 0.08 µg/ml and 0.016 µg/ml; Beckman Coulter, Austria) with PBS^+ buffer were used to confirm cell responsiveness. PBS^- buffer served as negative control to measure CD63 expression in absence of stimuli. 100 µl -aliquots of stained whole blood where added to 100 µl of each stimulus- and control-dilutions and incubated at 37°C for 40 minutes. To terminate the reaction, 100 µl 3.8% EDTA in aqua destillata were added to each sample. Lysis of erythrocytes occurred with 2 ml of a 1:10 dilution of lysis buffer in aqua destillata (8.3 % NH_4Cl, 1 % CO_3, 37.2 mg Na2EDTA/100 ml in aqua destillata) and incubation in the dark at room temperature for 15 minutes. After centrifugation at 200 g for 5 minutes the cell samples were washed with 3 ml of PBS- buffer each followed by centrifugation at 200 g for 5 minutes. The cells were re-suspended in 400 µl cell fixation buffer (1:80 dilution of BD Cell fix, Beckman Coulter with PBS^- buffer) and analyzed by three-color flow cytometry (FC500, Beckman Coulter). (30,56)

Determination of allergen-induced basophil activation through quantification of cellular CD63 up-regulation was performed as previously described by Sturm G.J. et al. 2004 (see figure 7): Basophil granulocytes were identified as a single population of cells expressing CD123 ($CD123^+$), but not HLA-DR ($HLA\text{-}DR^-$) (figure 7A). For each sample 300 basophil

target events were acquired. CD63 expression was determined through FITC fluorescence (anti-CD63 FITC-conjugated antibody), where an increase in fluorescence indicated CD63 up-regulation. Quantification of CD63 up-regulation occurred via the percentage of basophils found in a region of high FITC fluorescence (C2). For the negative control C2 was adjusted to contain 10% of basophils representing the portion of activated basophils in an unstimulated sample (figure 7B). In the C2 region a more than 2.5-fold increase in basophils as compared to the negative control was considered a positive response. Figure 7C shows basophil activation for a patient allergic to wasp venom. (30)

Figure 7: Quantification of allergen-induced CD63 up-regulation as marker for cellular activation in basophil granulocytes



Basophil granulocytes were identified as a single population of $CD123^+$ and $HLA\text{-}DR^-$ cells (A). Quantification of CD63 up-regulation occurred via a region of high FITC fluorescence (C2). For the negative control (B) it was adjusted to contain 10% of basophils accounting for the portion of activated basophils in an un-stimulated sample. A more than 2.5-fold increase of basophils in C2 was considered a positive response, such as seen for a sample of a wasp-allergic patient that showed 74% of basophil activation after stimulation with wasp venom.(C). (Previously described by (30)).

For the second protocol the commercially available assay Flow 2 Cast® from Bühlmann Laboratories (Schönenbuch, Switzerland) was performed according to the manufacturer's instructions.

3.2.7 Sting challenge test

Insect sting challenge tests were performed on patients with double sensitization to bee and wasp venom in IDT and CAP and mono-sensitization in one of the two BAT-protocols. For the sting challenge test living bees (Apis melifera) and wasps (V. germanica, V.vulgaris)

were used that were supplied by the Institute of Zoology Graz and by a local pest control service, respectively. Testing occurred under partial inpatient conditions and intensive medical stand-by; vital signs (systolic and diastolic blood pressure, pulse, arterial oxygen saturation), ECG and pulmonary function of the patients were monitored. The sting was performed on the forearm of the patient. To confirm the ability of BAT to correctly determine the clinically irrelevant insect venom, insects whose venom tested negative in the BAT were used for the sting challenge. Since quantity and composition of the venom released during the sting could not be determined, only sting challenge test resulting in a clear local reaction (see section 1.3.1.) were evaluated.

3.2.8 Clinical laboratory tests

A venous blood sample of 15 ml was collected for chemistry, coagulation and hematology assessments. The clinical laboratory tests were performed by the routine laboratory.

3.3 Statistics

Data was analyzed as median, 25^{th} and 75^{th} percentile unless indicated otherwise. sIgE and sIgG4 levels, sIgE:sIgG4 ratio and the specific activity were analyzed by Wilcoxon signed-rank test.

4 Results

Patients with a history of at least grade II systemic anaphylactic reactions (according to Ring and Messmer) who showed double-sensitization in intradermal skin testing (IDT) as well as in sIgE quantification (CAP) were included. For equivocal results in standard testing, the application of cellular testing systems is recommended (27). The current study focuses on the basophil activation test (BAT) and its ability to identify the clinically irrelevant insect venom. Thus, patients' sera were further analyzed by two different BAT protocols. Negative test results in one of the two protocols were confirmed by sting challenge tests with the negative insects.

4.1 History and demographic data

The study population was composed of 14 patients ranging from 19 to 63 years of age with a median age of 44.5 years (35.25; 48.5). It consisted of 50% (7/14) male and 50% (7/14) female individuals who had a history of anaphylactic reactions grade ≥ II according to Ring and Messmer. 57.1% (8/14) experienced grade II reactions and 42.9% (6/14) grade III reactions. 14% (2/14) identified bees, 43% (6/14) wasps and 14% (2/14) hornets as culprit insects. 29% (4/14) were not able to determine the insects responsible for the experienced systemic reactions.

4.2 Sting challenge testing

Individuals included into the study were analyzed with two different BAT protocols, referred to as BAT Graz and BAT Bühlmann, where mono-sensitization in one of them served as basis for choosing insects for the subsequent sting challenges. Table 8 gives an overview over the patients' BAT testing results as well as a summary of the sting challenge tests.

Table 8: Overview of the patients' testing results

Patient	BAT Graz		BAT Bühlmann		Conclusion: BAT	Sting challenge	
ID	*Bee*	*Wasp*	*Bee*	*Wasp*	*Mono-sensitization*	*Insect*	*Reaction*
1		+		+	wasp	bee	LLR
2	+	+		+	wasp	bee	NLR
3		+		+	wasp	bee	NLR
4	+	+	+		bee	wasp	NLR
5		+		+	wasp	bee	LLR
6	+	+		+	wasp	bee	NLR

7		+	+	wasp	bee	LLR
8		+	+	wasp	bee	NLR
9	+	+	+	wasp	bee	LLR
10		+	+	wasp	bee	SSR (G1)
11		+	+	wasp	bee	LLR
12		+	+	wasp	bee	NLR
13	+	+	+	wasp	bee	LLR
14	+	+	+	wasp	bee	LLR

For the BAT protocols, BAT Graz and BAT Bühlmann, positive test results for bee and wasp venoms are indicated (+). The sting challenge shows the insects used in each test and the patient's reaction to it, either a normal local reaction (NLR), a large local reaction (LLR) or a systemic sting reaction (SSR), where its severity (Gx) is graded according to Ring and Messmer

Where previous IDT and CAP indicated double-sensitization, a combination of two BAT protocols was able to determine mono-sensitization in all 14 (100%) study individuals. Concluding from the BAT tests, 13 (93%) of them showed mono-sensitization to wasp venom and one to bee venom. In the sting challenges, insects were used whose venom did not elicit basophil activation, so that one patient was exposed to the sting of a wasp and 13 (93%) patients to that of bees. 6 of 14 patients (43%) experienced normal local reactions, 7 (50%) large local reactions and in one patient a systemic reaction of grade I was observed. The systemic reaction occurred 30 minutes after the sting of a bee and manifested in a wheal (urtica) at the patients neck and two wheals at the border of a large local reaction. In addition, an exanthema developed one hour after the sting.

The study population can thus be divided into three different study groups: the two populations that tested negative for bee or wasp venom in the BAT and did not show systemic reactions during sting challenges with the negative insects. (Referred to in the following as "BAT(bee$^-$) no SSR" and "BAT(wasp$^-$) no SSR", respectively) And the group of patients where no reactivity to bee venom could be observed in the BAT, but the subsequent sting challenge with a bee resulted in a systemic reaction. (Referred to in the following as "BAT(bee$^-$) SSR")

4.3 Comparison of BAT protocols

Patents' sera were analyzed with two different protocols of BAT: One protocol was commercially available from Bühlmann Laboratories (Schönenbuch, Switzerland), referred

to as BAT Bühlmann, and the other protocol was developed by the Department of Dermatology and Venerology at the LKH University Hospital of Graz, referred to as BAT Graz. The results of the BAT testing can be seen in table 8 and 9.

Table 9: Comparison of the two BAT protocols via their test results and the negative predictive values resulting from them

	BAT Graz		BAT Bühlmann	
Mono-sensitization	57%	8/14	100%	14/14
Double-sensitization	43%	6/14	0%	0/14
Negative predictive value	87.5%		92.9%	

The BAT Graz was able to determine mono-sensitization in the majority of the analyzed samples, where IDT and CAP yielded double-sensitization. 1 of 8 patients with mono-sensitization in the BAT Graz experienced a systemic anaphylactic reaction of grade I when stung by the determined negative insect. For 7 of 8 (87.5%) study subjects the BAT Graz was able to correctly identify the clinically irrelevant insect venom. The negative predictive value of the BAT Graz was thus found to be 87.5%.

In comparison, in all analyzed samples the BAT Bühlmann detected mono-sensitization to either bee or wasp venom where standard testing indicated double-sensitization to both venoms. When stung by the insect tested negative in the BAT Bühlmann 1 of 14 patients showed a grade I- anaphylactic reaction, whereas for 13 (92.9%) of them the clinically irrelevant insect was correctly determined. The negative predictive value of the BAT Bühlmann was thus calculated 92.9%.

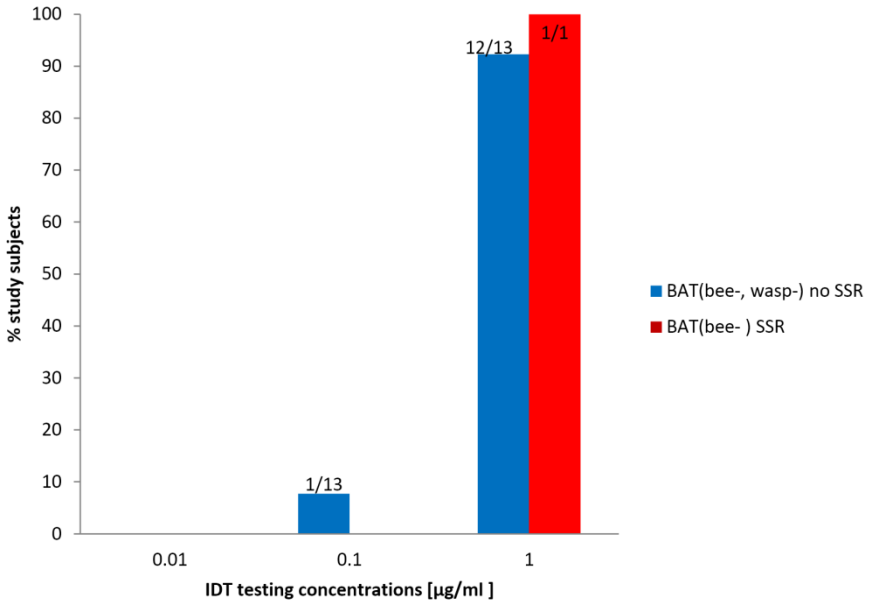
4.4 Comparison of IDT, CAP, CRD and screening for CCD

Results of IDT and CAP including CRD and screening for CCD can be analyzed with respect to the previously defined three study groups.

4.4.1 Double-sensitization

All patients included into the study showed double-sensitization to bee and wasp venom in IDT and CAP. Figure 8 and 9 analyze sensitization levels to insect venoms that tested negative in BAT. To analyze differences between the patient that experienced a systemic reaction during the sting challenge and patients that did not, the groups "BAT(bee$^-$) no SSR" and "BAT(wasp$^-$) no SSR" were combined into one group, referred to as "BAT(bee$^-$, wasp$^-$) no SSR".

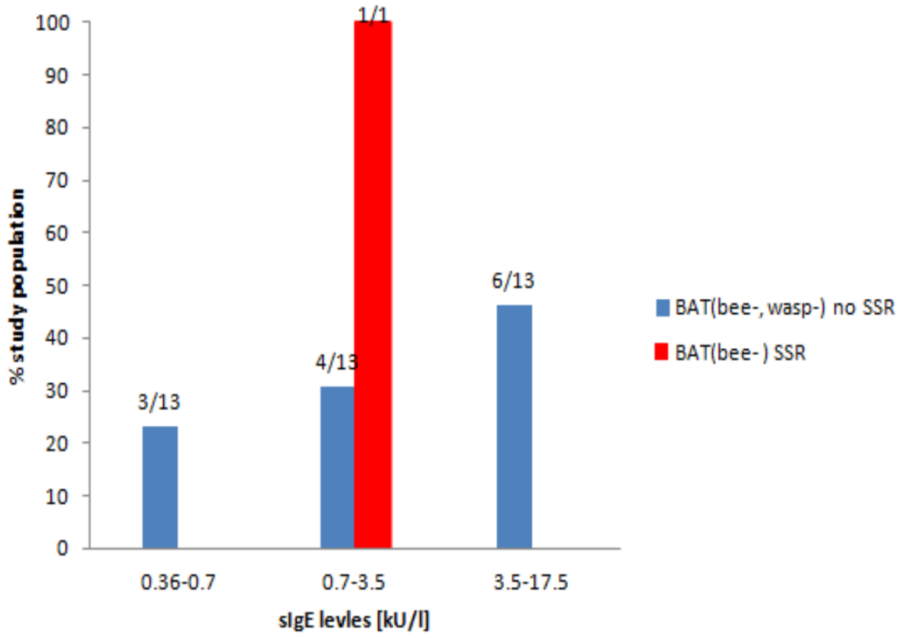
Figure 8: Frequency of sensitization to the BAT negative insect venom in IDT:



Shown are the lowest venom concentrations where sensitization to the BAT negative insect venom was observed.

"BAT(bee$^-$) SSR" and "BAT(bee$^-$, wasp$^-$) no SSR" indicate sensitization levels of patients that did and did not experience systemic reactions during the sting challenge, respectively.

Figure 9: Frequency of sensitization to the BAT negative insect venom in sIgE quantification



Displayed are sIgE levels against the BAT negative insect venoms and their distribution over different ranges of antibody levels. sIgE levels above 0.35 kU/l were considered to indicate sensitization. "BAT(bee$^-$) SSR" and "BAT(bee$^-$, wasp$^-$) no SSR" indicate sIgE levels of patients that did and did not experience systemic reactions during the sting challenge, respectively.

For patients that did not react systemically during the sting challenge (BAT(bee$^-$, wasp$^-$) no SSR), results of the BAT were confirmed showing that sensitization to either bee or wasp venom, as detected in IDT and CAP, was asymptomatic. In IDT, the majority of them (92%) showed sensitization to the BAT negative insect at the highest venom concentration used in testing (1 µg/ml). Concerning CAP, test results were grouped according to the measured sIgE levels. Levels exceeding 0.35 kU/l were considered positive and indicated sensitization. Most patients (46%) showed sensitization to the BAT negative insect at high sIgE levels exceeding 3.4 kU/l.

Concerning the "BAT(bee$^-$) SSR" patient, BAT indicated bee venom to be clinically irrelevant; the subsequent sting challenge did not confirm this result. For this patient IDT and CAP showed sensitization to bee venom at a venom concentration of 1µg/ml and at sIgE levels of 1.34 kU/l, respectively.

4.4.2 Sensitization in CRD

Specific IgE levels against recombinant allergens of the BAT negative insect venoms were of interest (table 10).

Table 10: Sensitization to recombinant allergens of the BAT negative insect venoms

Study group	Sensitization		No- Sensitization	
	Recombinant Allergen	Frequency	Recombinant Allergen	Frequency
BAT(bee$^-$) no SSR	rApi m 1	1/12	rApi m 1	11/12
BAT(bee$^-$) SSR	-	-	rApi m 1	1/1
BAT(wasp$^-$) no SSR	rVes v 1	1/1	rVes v 5	1/1

Shown are recombinant allergens (rApi m 1, rVes v 1, rVes v 5) of the BAT negative insect venoms and the frequency at which sensitization to them was observed.

The three study groups are defined as "BAT(bee$^-$) no SSR", "BAT(wasp$^-$) no SSR" and "BAT(bee$^-$) SSR", where BAT(x$^-$) indicates the BAT negative insect venom detected for this group and "no SSR" and SSR" the occurrence of a systemic sting reaction (SSR) when exposed to this venom during a sting challenge.

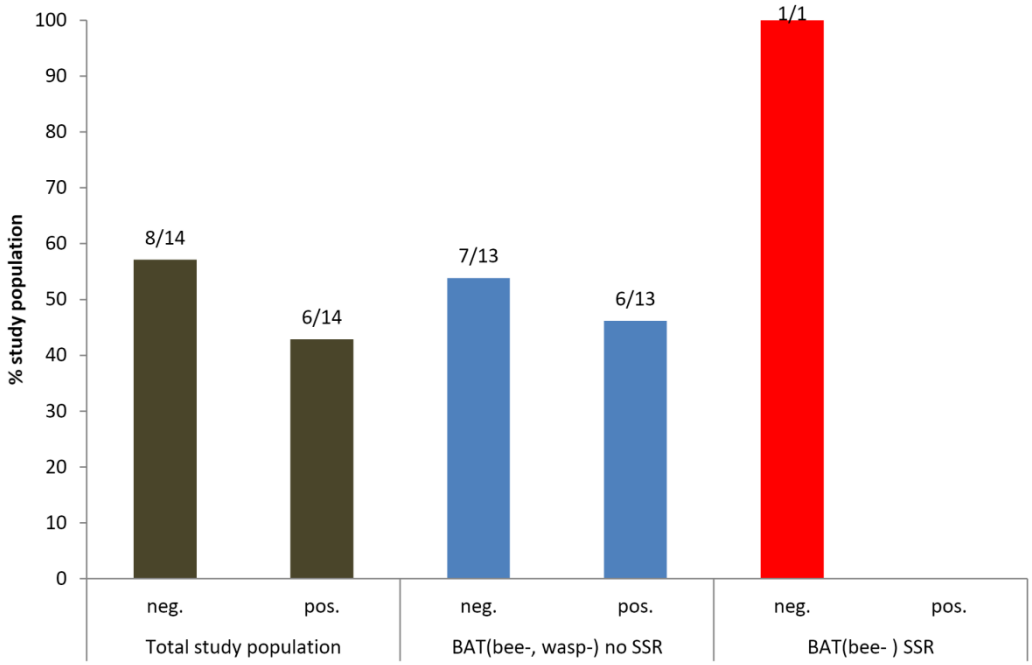
sIgE levels against rApi m 1 were analyzed for the study groups "BAT(bee$^-$) no SSR" and "BAT(bee$^-$) SSR". Only one of them showed sensitization to this allergen, while the majority (92%) tested negative. Interestingly, the patient who reacted systemically to the bee sting challenge did not show sensitization to rApi m 1.

The recombinant allergens rVes v 1 and rVes v 5 were analyzed for the "BAT(wasp$^-$) no SSR" patient. In this patient sensitization to rVes v 1 but not to rVes v 5 was detected.

4.4.3 Presence of CCD

sIgE against CCD may interfere with sIgE quantification to purified bee and wasp venom simulating double-sensitization. As all study subjects showed double-sensitization in CAP, the presence of anti-CCD-sIgE antibodies in their sera was analyzed. (figure 10).

Figure 10: Presence of anti-CCD-sIgE antibodies



Indicated are the total study population as well as patients that did (BAT(bee$^-$) SSR) and did not (BAT(bee$^-$, wasp$^-$) no SSR) experience systemic reactions during the sting challenge.

In the majority (57%) of the total study population no anti-CCD sIgE antibodies could be detected. Of the patients that did not show systemic reactions during the sting challenge 46% showed positive anti-CCD-sIgE levels while in most of them cross-reactivity due to CCD could be excluded. For the patient that experienced a systemic reaction during the sting challenge anti-CCD sIgE levels were negative.

4.5 Positive sting challenge

During the sting challenges one patient out of 14 experienced a mild grade I - anaphylactic reaction when exposed to the sting of a bee. The systemic reaction occurred 30 minutes after the sting of a bee and manifested in a wheal (urtica) at the patients neck and two wheals at the border of a large local reaction. In addition, an exanthema developed one hour after the sting.

In his anaphylactic history the patient reported one incident, where he was exposed to over 100 stings of supposedly wild wasps. The stings occurred on his head, legs and arms. Symptoms manifested within 10 minutes including intense pain in the soles of his feet, visual impairments, such as flickering and perceiving his environment in shades of yellow and grey, and erythrodermia. The patient lost consciousness for several minutes.

In the foregoing tests with two BAT protocols, both clearly indicated mono- sensitization to wasp venom and determined bee venom as clinically irrelevant (table 8). In IDT sensitization to bee venom was detected at a concentration of 1µg/ml as for 92% of patients that did not react systemically in the sting challenge (figure 8). sIgE levels against purified bee venom were elevated with 1.34 kU/l, however 77% of patients that did not react systemically showed levels similar or even higher than that (figure 9). Sensitization to rApi m 1 was not observed, cross-reactivity due to CCD could be excluded.

Patient's serum tryptase levels before the challenge were 2.4 µg/L and 2.3 µg/L 3 hours after the sting.

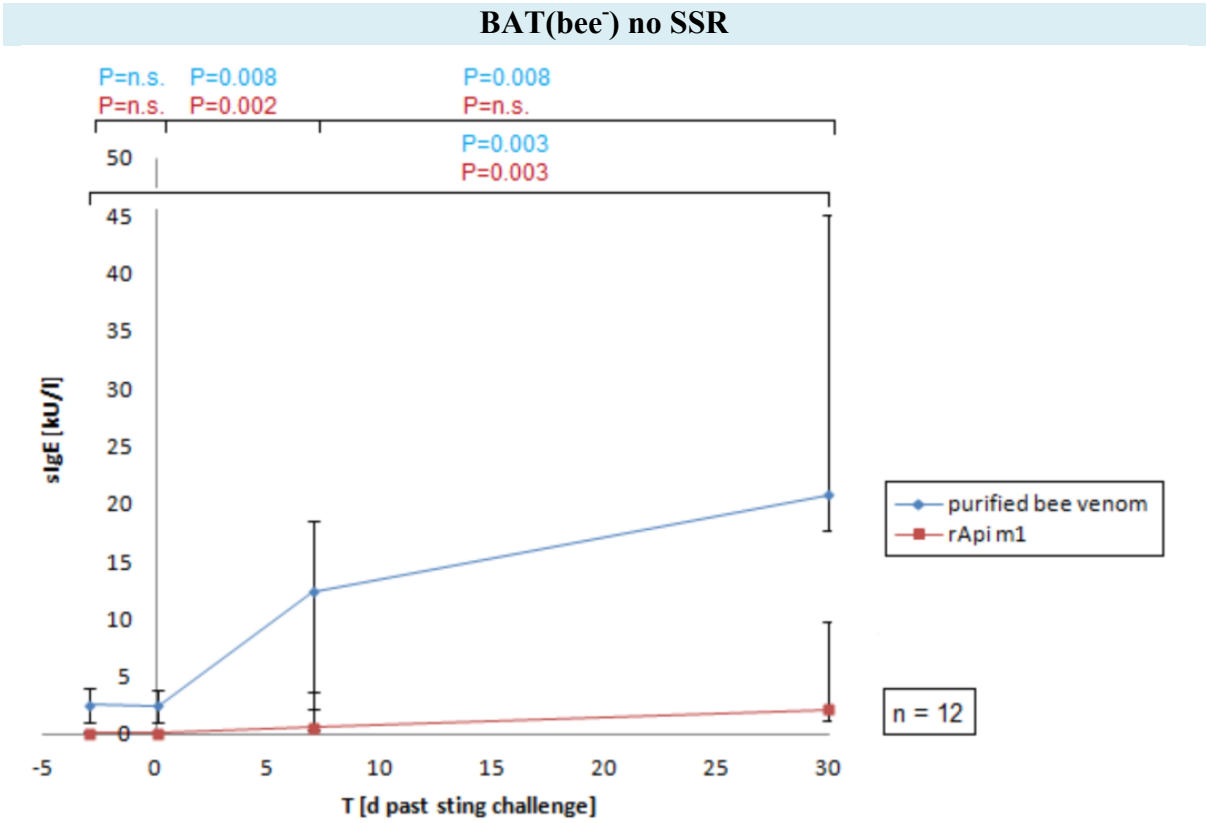
4.6 sIgE and sIgG4 level, ratio sIgE:sIgG4 and specific activity

The time course of sIgE and sIgG4 levels, of the sIgE:sIgG4 ratios as well as of the IgE-specific activities were analyzed for the three study groups.

4.6.1 Time course of sIgE levels

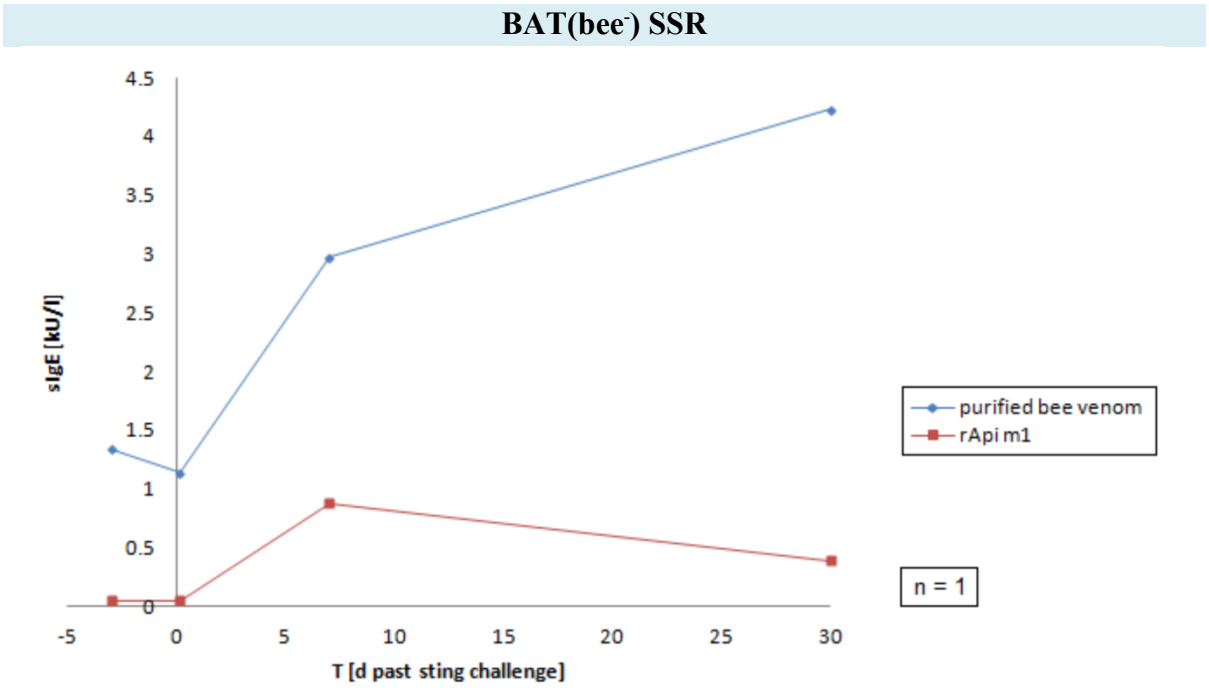
4.6.1.1 sIgE: bee venom and rApi m 1

Figure 11: Time course of sIgE levels against bee venom and rApi m 1 of the "BAT(bee⁻) no SSR" study group



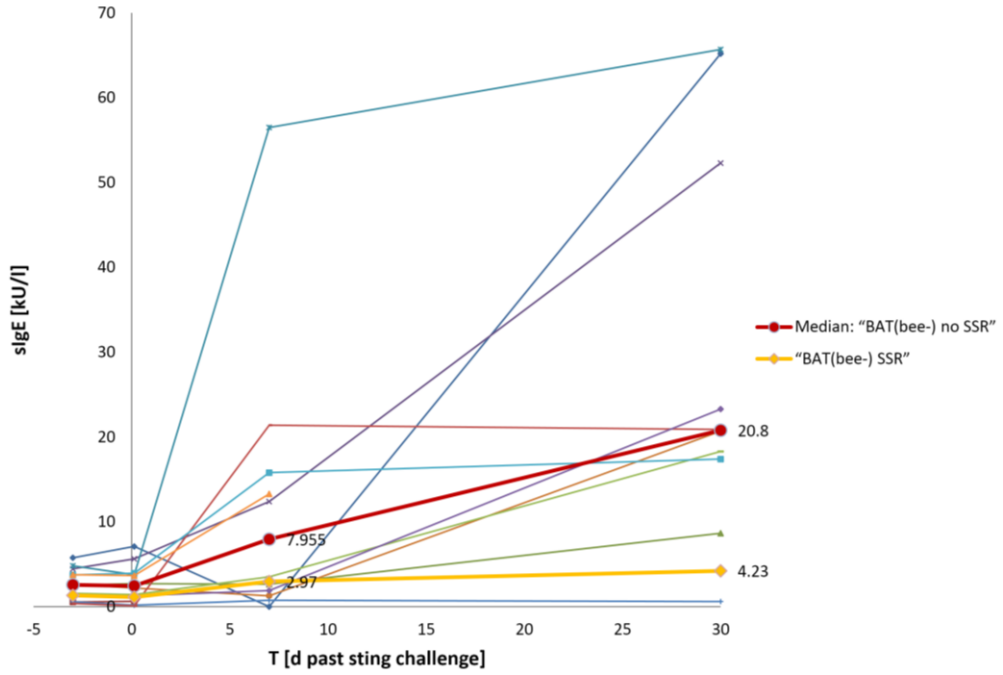
The "BAT(bee⁻) no SSR" group represents all study individuals for whom bees were detected as BAT negative insect and who did not show systemic reactions when challenged with the sting of a bee. The study group comprised 12 patients. T = 0 indicates the point of sting challenge with a bee. sIgE levels against bee venom and rApi m 1 were analyzed prior to, 3 hours, 7 and 30 days after the challenge.

Figure 12: Time course of sIgE levels against bee venom and rApi m 1 of the "BAT(bee⁻) SSR" study individual



One patient for whom bees were determined as BAT negative insect showed a systemic anaphylactic reaction in the bee sting challenge, referred to as "BAT(bee⁻) SSR". T = 0 indicates the point of sting challenge with a bee. sIgE levels against bee venom and rApi m 1 were analyzed prior to, 3 hours, 7 and 30 days after the challenge.

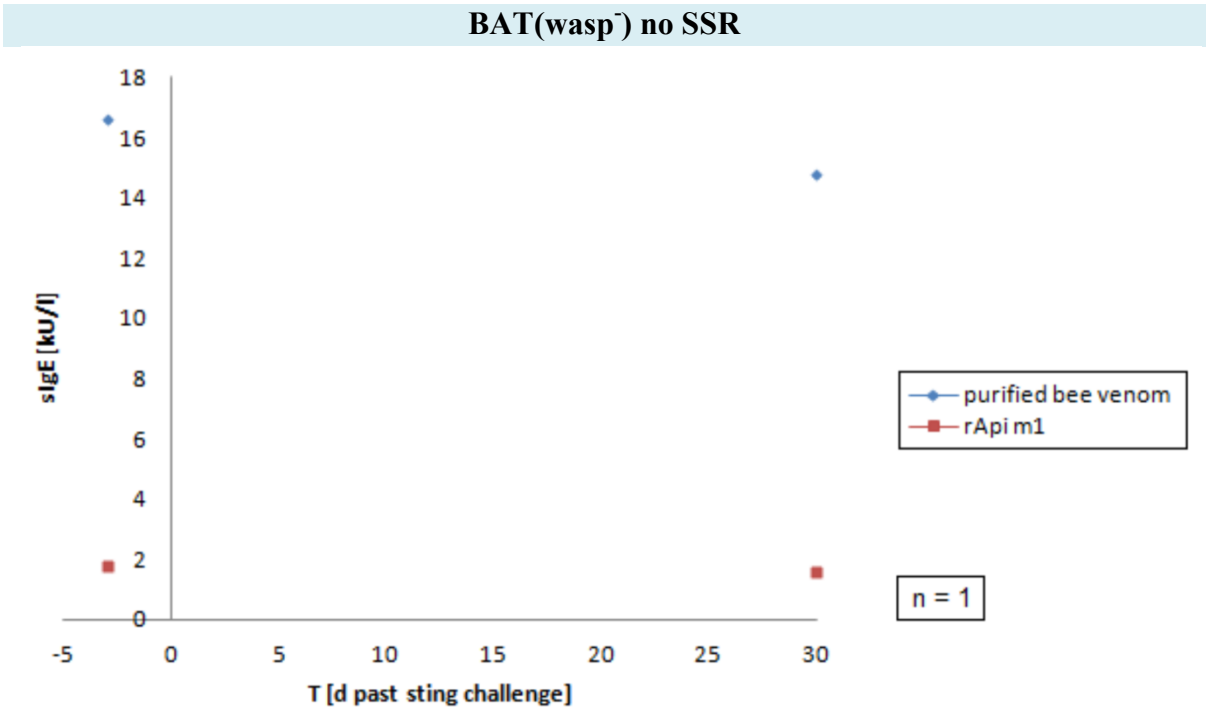
Figure 13: Time course of sIgE levels against bee venom: "BAT(bee⁻) no SSR" vs. "BAT(bee⁻) SSR"



Comparison of the time course of sIgE levels against bee venom for all study patients that were challenged with the sting of a bee. T = 0 represents the point of sting challenge. The median (red) of the

"BAT(bee⁻) no SSR" group and the "BAT(bee⁻) SSR" patient (yellow) are highlighted, where "BAT(bee⁻) SSR" represents the only study individual that experienced a systemic anaphylactic reaction grade I to the bee sting challenge.

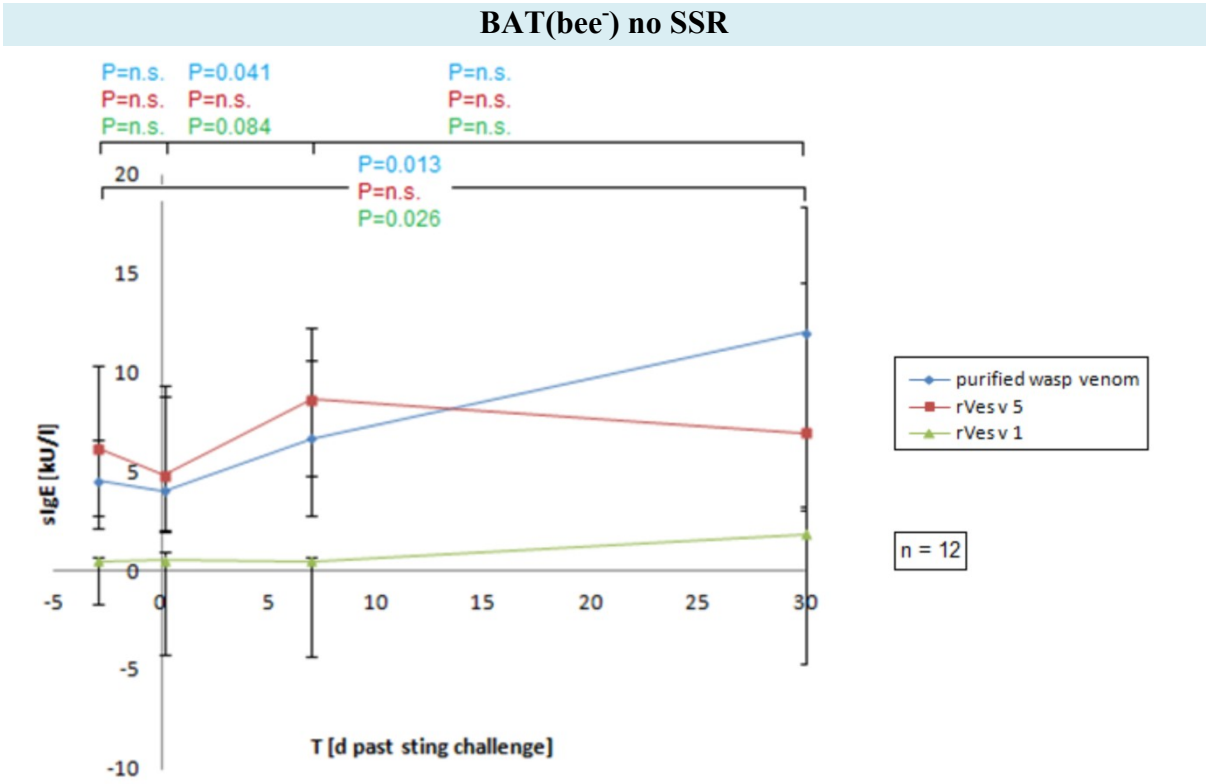
Figure 14: Time course of sIgE levels against bee venom and rApi m 1 of the "BAT(wasp⁻) no SSR" study individual



For one patient wasps were determined as BAT negative insect; when exposed to the sting of a wasp no systemic reaction was triggered, referred to as "BAT(wasp⁻) no SSR". T = 0 indicates the point of sting challenge with a wasp. sIgE levels against bee venom and rApi m 1 were analyzed prior to and 30 days after the challenge.

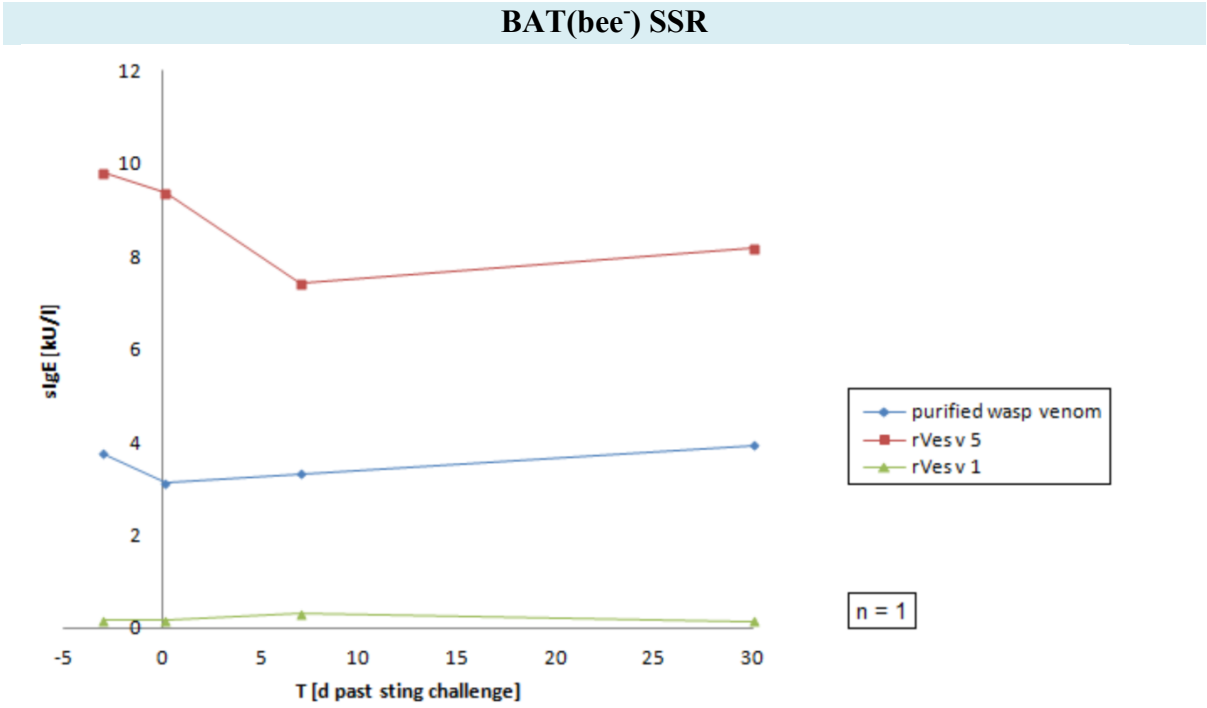
4.6.1.2 sIgE: wasp venom, rVes v 1 and rVes v 5

Figure 15: Time course of sIgE levels against wasp venom, rVes v 1 and rVes v 5 of the "BAT(bee⁻) no SSR" study group



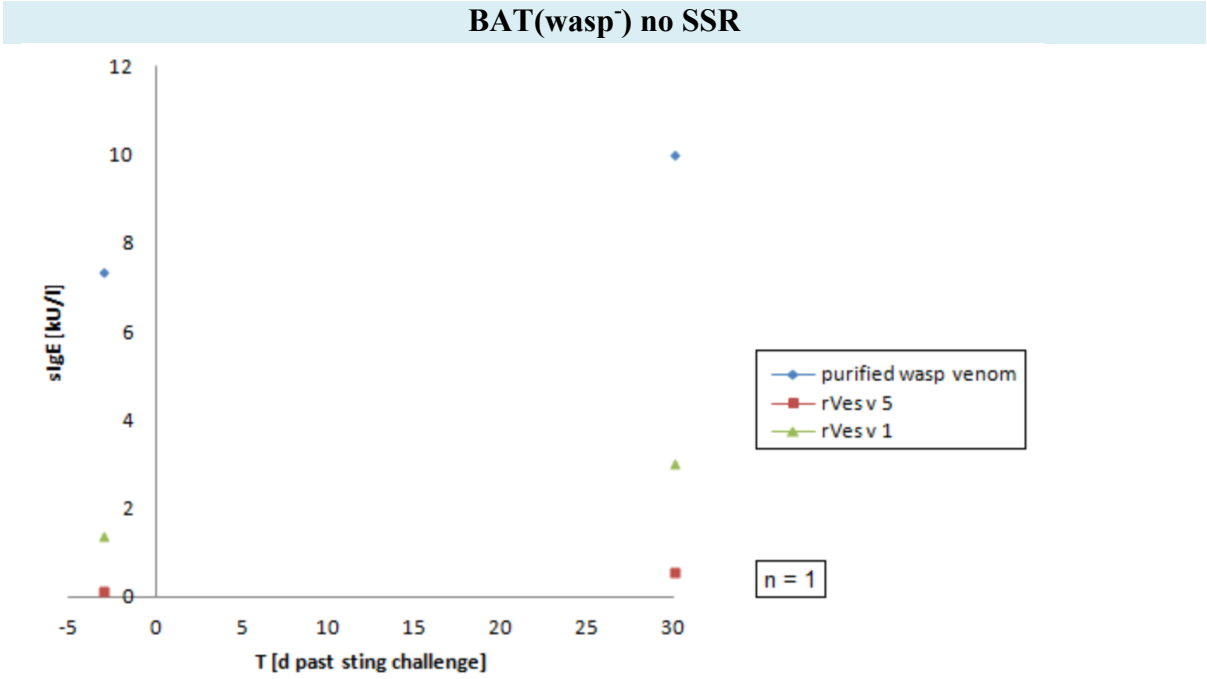
The "BAT(bee⁻) no SSR" group represents all study individuals for whom bees were detected as BAT negative insect and who did not show systemic reactions when challenged with the sting of a bee. The study group comprised 12 patients. T = 0 indicates the point of sting challenge with a bee. sIgE levels against wasp venom, rVes v 1 and rVes v 5 were analyzed prior to, 3 hours, 7 and 30 days after the challenge.

Figure 16: Time course of sIgE levels against wasp venom, rVes v 1 and rVes v 5 of the "BAT(bee$^-$) SSR" study individual



One patient for whom bees were determined as BAT negative insect showed a systemic anaphylactic reaction in the bee sting challenge, referred to as "BAT(bee$^-$) SSR". T = 0 indicates the point of sting challenge with a bee. sIgE levels against wasp venom, rVes v 1 and rVes v 5 were analyzed prior to, 3 hours, 7 and 30 days after the challenge.

Figure 17: Time course of sIgE levels against wasp venom, rVes v 1 and rVes v 5 of the "BAT(wasp$^-$) no SSR" study individual



For one patient wasps were determined as BAT negative insect; when exposed to the sting of a wasp no systemic reaction was triggered, referred to as "BAT(wasp$^-$) no SSR". T = 0 indicates the point of sting challenge with a wasp. sIgE levels against wasp venom, rVes v 1 and rVes v 5 were analyzed prior to and 30 days after the challenge.

sIgE levels determine sensitization to a particular allergen. Subjects of the "BAT(bee$^-$) no SSR" study group were challenged with the sting of a bee. Compared to the pre-sting situation sIgE levels remained unchanged 3 hours after the sting. 7 days later, a significant increase in sIgE against purified bee venom and rApi m 1 was observed. From that point to 30 days after the sting another significant rise in sIgE against bee venom was found. Over the whole course of the study, starting from the pre-challenge situation to 30 days after the sting, sIgE levels against both bee venom and rApi m 1 increased significantly. (figure 11) This increase in sIgE was to be expected, as a stinging incident generally stimulates sIgE production against allergens of that venom. Interestingly, also a significant increase in sIgE levels against purified wasp venom and rVes v 1 could be noticed (figure 15).

For the "BAT(bee$^-$) SSR" patient, an initial decease of the sIgE level against bee venom was found 3 hours after the challenge, while it rose in the following course of the study. Overall a noticeable increase in sIgE against bee venom could be seen (figure 12). This increase was however not as prominent as that of most patients of the "BAT(bee$^-$) no SSR"

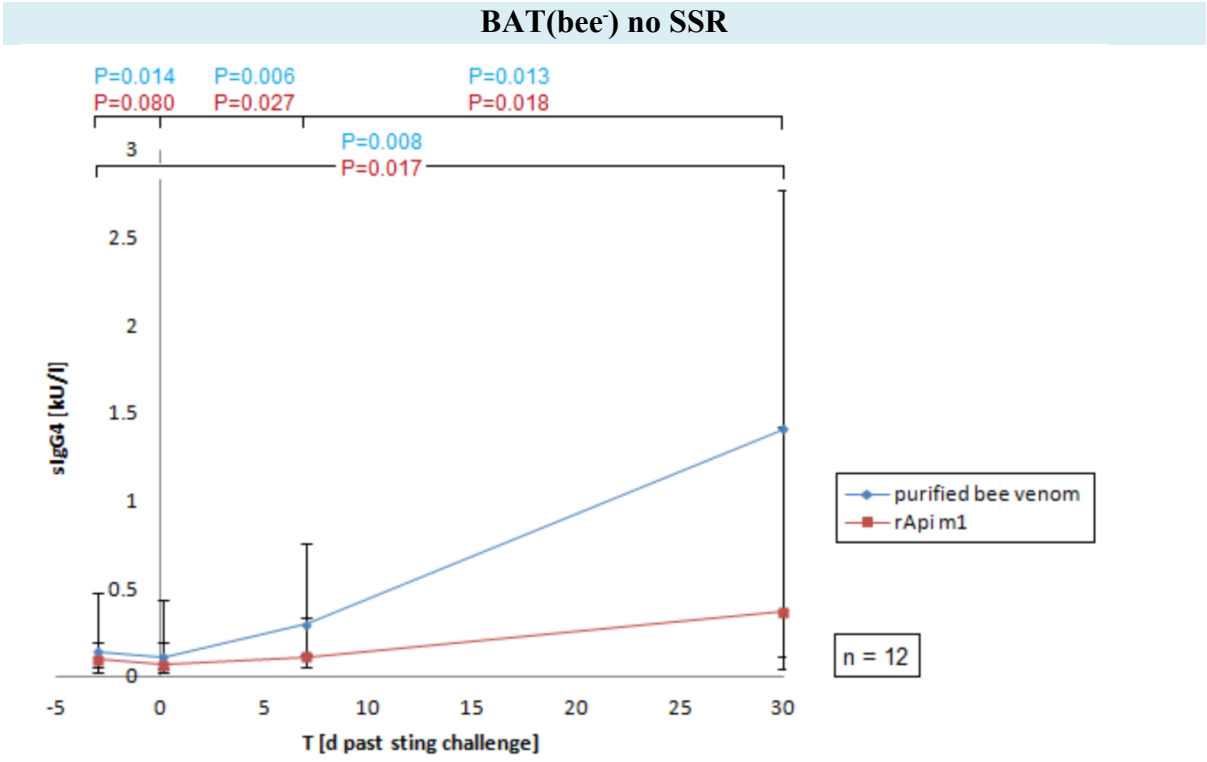
study group. (figure 13). The time course of sIgE against rApi m 1 seemed to differ from that of the BAT(bee$^-$) no SSR" group. 3 hours to 7 days after the challenge sIgE against rApi m 1 increased and decreased again over the remaining course of the study. Since there was only one patient who reacted systemically, no statement about the significance of these developments could be made.

For the "BAT(wasp$^-$) no SSR" patient an increase in sIgE against wasp venom, rVes v 1 and rVes v 5 could be observed 30 days after the sting (figure 17). Also for this patient its significance could not be evaluated.

4.6.2 Time course of sIgG4 levels

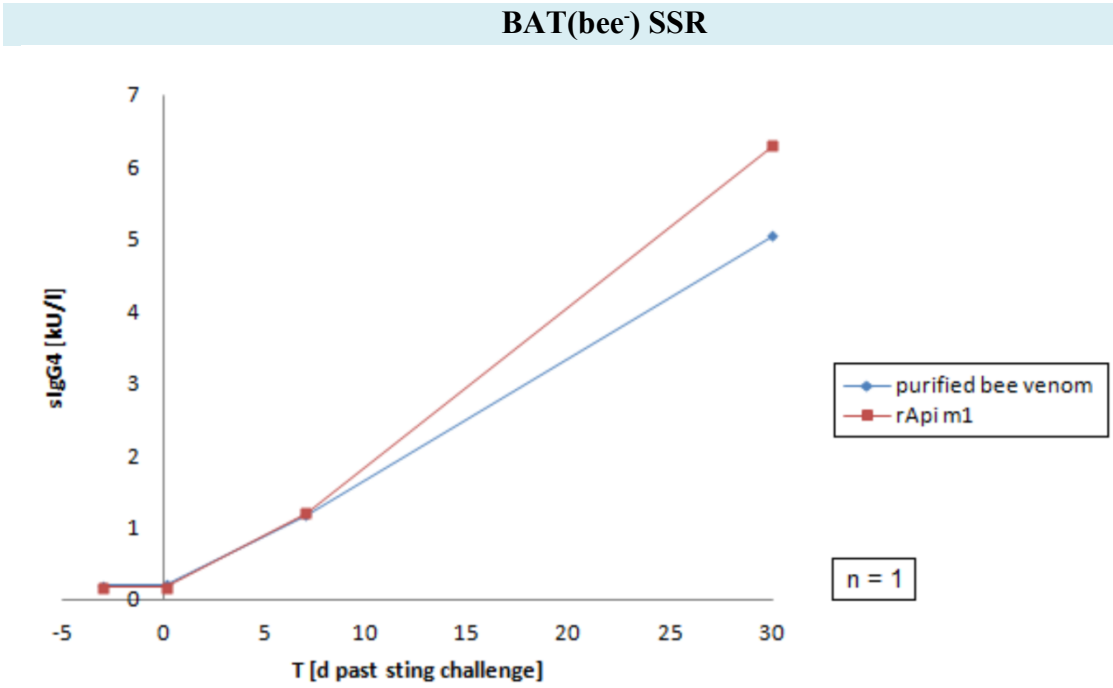
4.6.2.1 sIgG4: bee venom and rApi m 1

Figure 18: Time course of sIgG4 levels against bee venom and rApi m 1 of the "BAT(bee$^-$) no SSR" study group



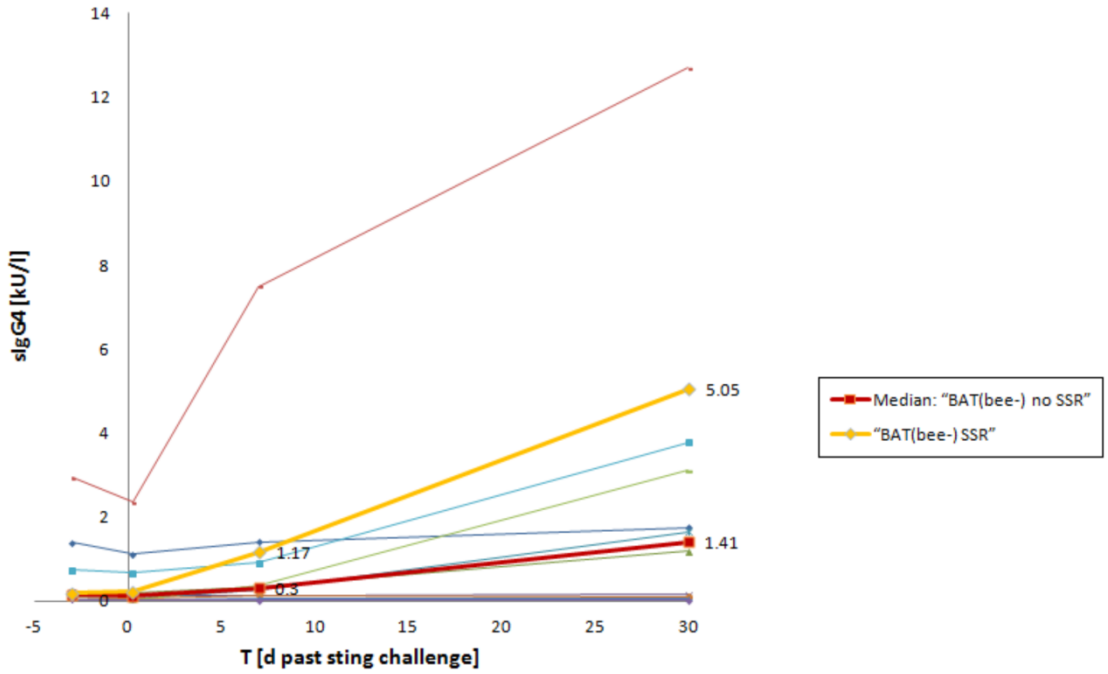
The "BAT(bee$^-$) no SSR" group represents all study individuals for whom bees were detected as BAT negative insect and who did not show systemic reactions when challenged with the sting of a bee. The study group comprised 12 patients. T = 0 indicates the point of sting challenge with a bee. sIgG4 levels against bee venom and rApi m 1 were analyzed prior to, 3 hours, 7 and 30 days after the challenge.

Figure 19: Time course of sIgG4 levels against bee venom and rApi m 1 of the "BAT(bee$^-$) SSR" study individual



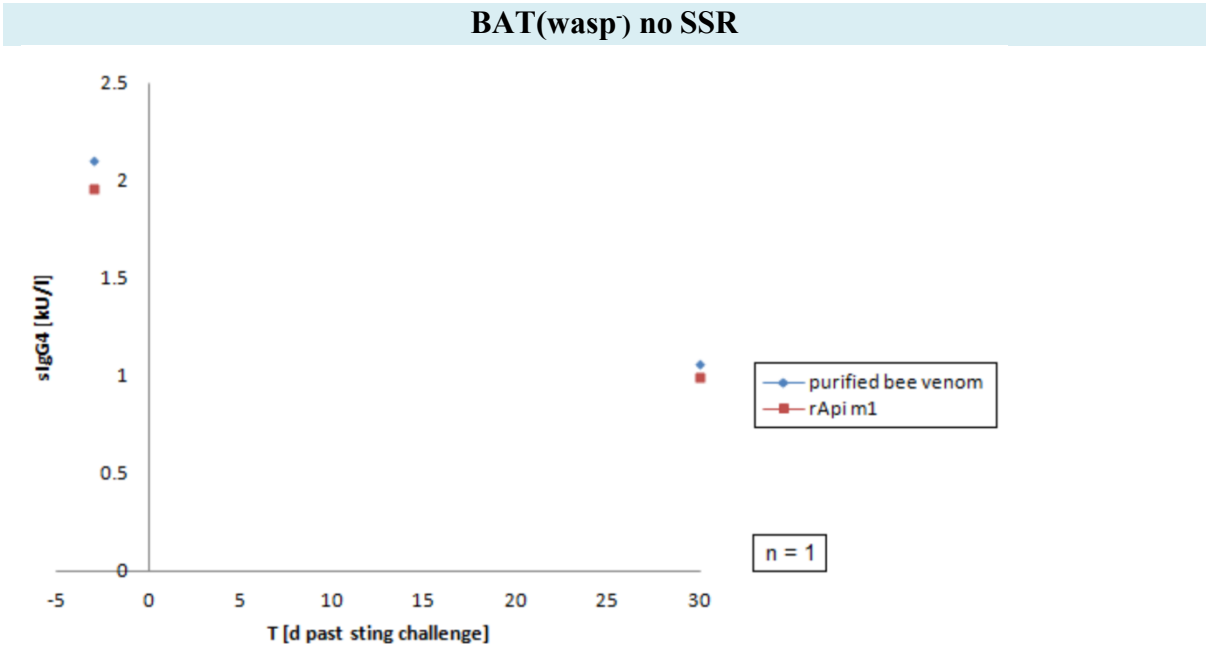
One patient for whom bees were determined as BAT negative insect showed a systemic anaphylactic reaction grade I in the bee sting challenge, referred to as "BAT(bee$^-$) SSR". T = 0 indicates the point of sting challenge with a bee. sIgG4 levels against bee venom and rApi m 1 were analyzed prior to, 3 hours, 7 and 30 days after the challenge.

Figure 20: Time course of sIgG4 levels against bee venom: "BAT(bee⁻) no SSR" vs. "BAT(bee⁻) SSR"



Comparison of the time course of sIgG4 levels against bee venom for all study patients that were challenged with the sting of a bee. T = 0 represents the point of sting challenge. The median (red) of the "BAT(bee⁻) no SSR" group and the "BAT(bee⁻) SSR" patient (yellow) are highlighted, where "BAT(bee⁻) SSR" represents the only study individual that experienced a systemic anaphylactic reaction grade I to the bee sting challenge.

Figure 21: Time course of sIgG4 levels against bee venom and rApi m 1 of the "BAT(wasp⁻) no SSR" study individual

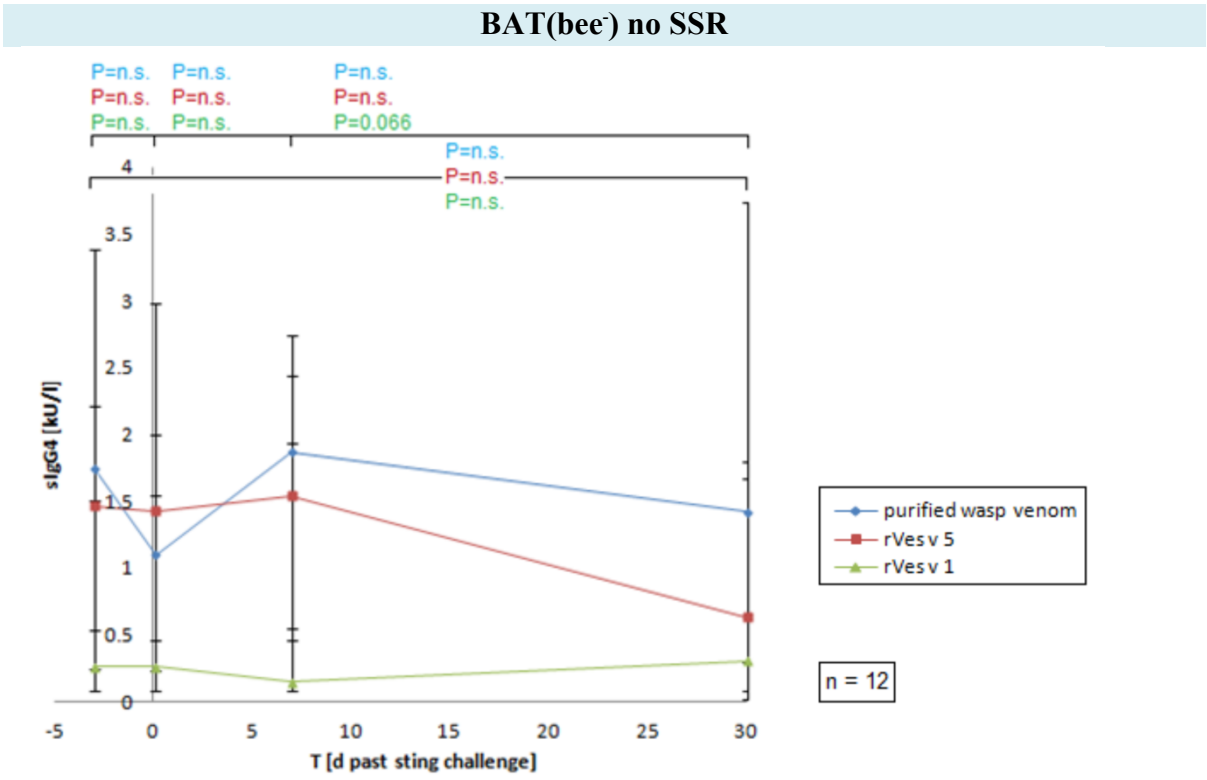


For one patient wasps were determined as BAT negative insect; when exposed to the sting of a wasp no systemic reaction was triggered, referred to as "BAT(wasp⁻) no SSR". T = 0 indicates the point of sting

challenge with a wasp. sIgG4 levels against bee venom and rApi m 1 were analyzed prior and 30 days after the challenge.

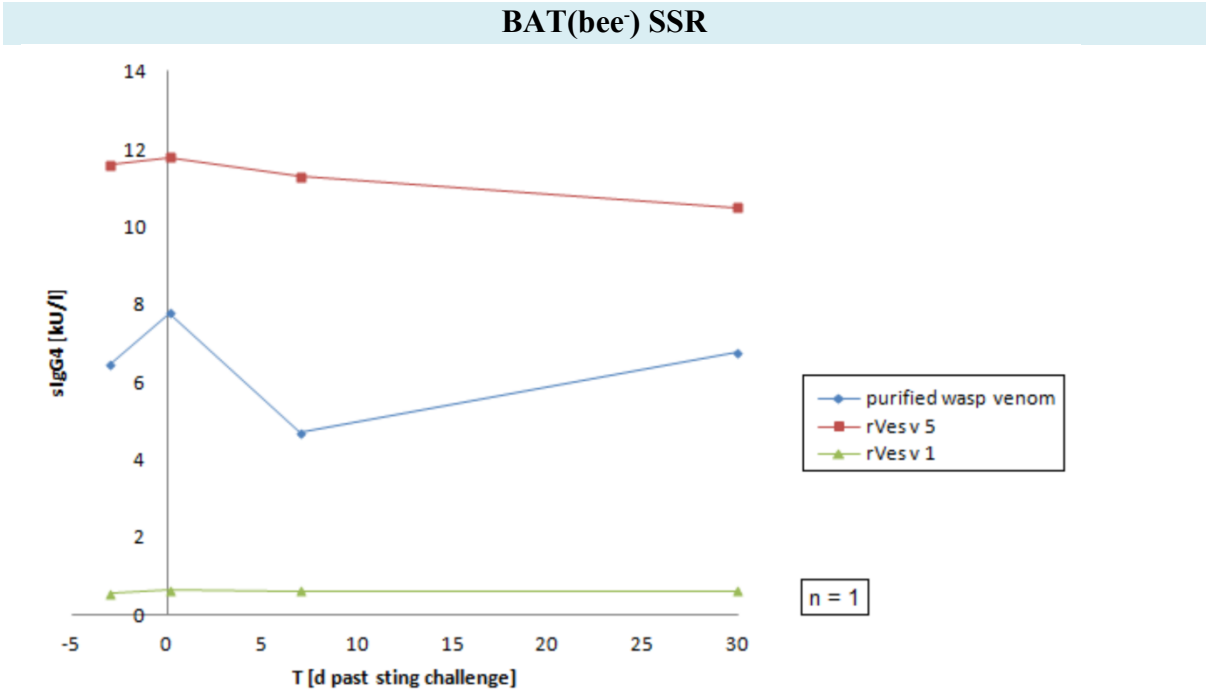
4.6.2.2 sIgG4: wasp venom, rVes v 1 and rVes v 5

Figure 22: Time course of sIgG4 levels against wasp venom, rVes v 1 and rVes v 5 of the "BAT(bee$^-$) no SSR" study group



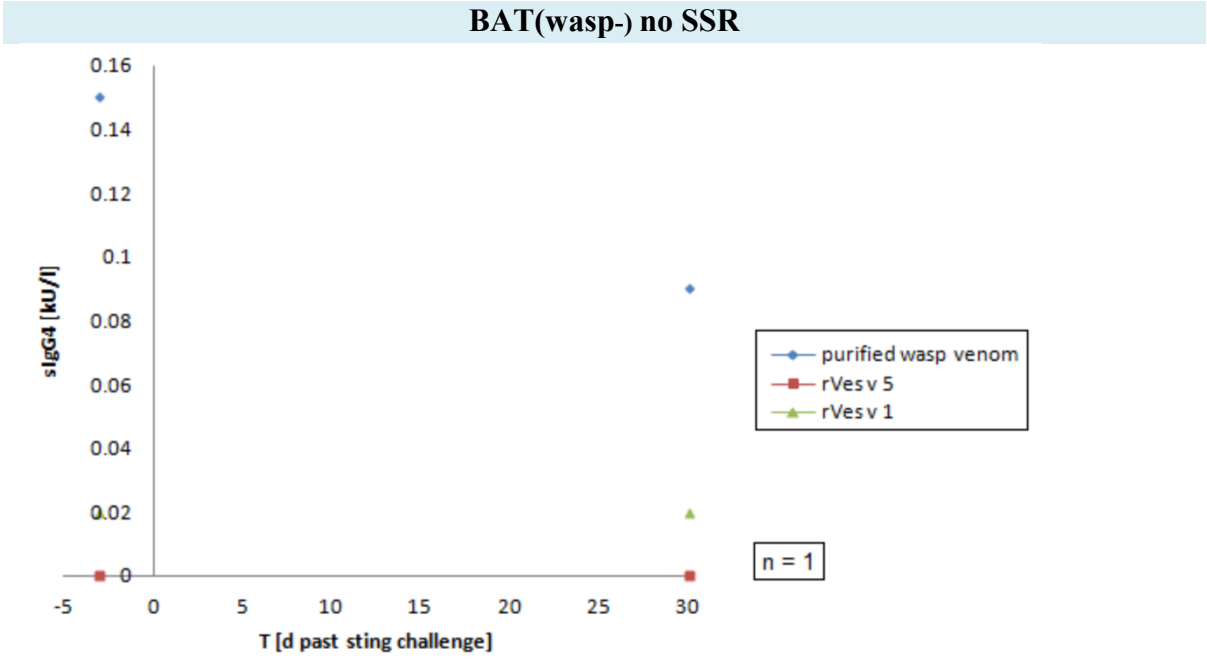
The "BAT(bee$^-$) no SSR" group represents all study individuals for whom bees were detected as BAT negative insect and who did not show systemic reactions when challenged with the sting of a bee. The study group comprised 12 patients. T = 0 indicates the point of sting challenge with a bee. sIgG4 levels against wasp venom, rVes v 1 and rVes v 5 were analyzed prior to, 3 hours, 7 and 30 days after the challenge.

Figure 23: Time course of sIgG4 levels against wasp venom, rVes v 1 and rVes v 5 of the "BAT(bee⁻) SSR" study individual



One patient for whom bees were determined as BAT negative insect showed a systemic anaphylactic reaction grade I in the bee sting challenge, referred to as "BAT(bee⁻) SSR". T = 0 indicates the point of sting challenge with a bee. sIgG4 levels against wasp venom, rVes v 1 and rVes v 5 were analyzed prior to, 3 hours, 7 and 30 days after the challenge.

Figure 24: Time course of sIgG4 levels against wasp venom, rVes v 1 and rVes v 5 of the "BAT(wasp⁻) no SSR" study individual



For one patient wasps were determined as BAT negative insect; when exposed to the sting of a wasp no systemic reaction was triggered, referred to as "BAT(wasp⁻) no SSR". T = 0 indicates the point of sting

challenge with a wasp. sIgG4 levels against wasp venom, rVes v 1 and rVes v 5 were analyzed prior to and 30 days after the challenge.

Specific IgG4 is considered a blocking antibody, thought to have some protective function against systemic allergic reactions. (39) In the "BAT(bee$^-$) no SSR" study group a significant reduction in sIgG4 levels against bee venom could be observed over the first 3 hours after the sting. sIgG4 levels against rApi m 1 showed a statistical trend of decreasing. Over the following 7 and 30 days the sIgG4 levels rose significantly so that over the whole study period a significant increase of sIgG4 against bee venom and rApi m1 was observed. (figure18) This may possibly show that sIgG4 blocking antibodies were used up during the immediate sting phase and were later, as a reaction to the sting, increasingly rebuild. sIgG4 levels against purified wasp venom, rVes v 1 and rVes v 5 did not show any significant changes (figure 22).

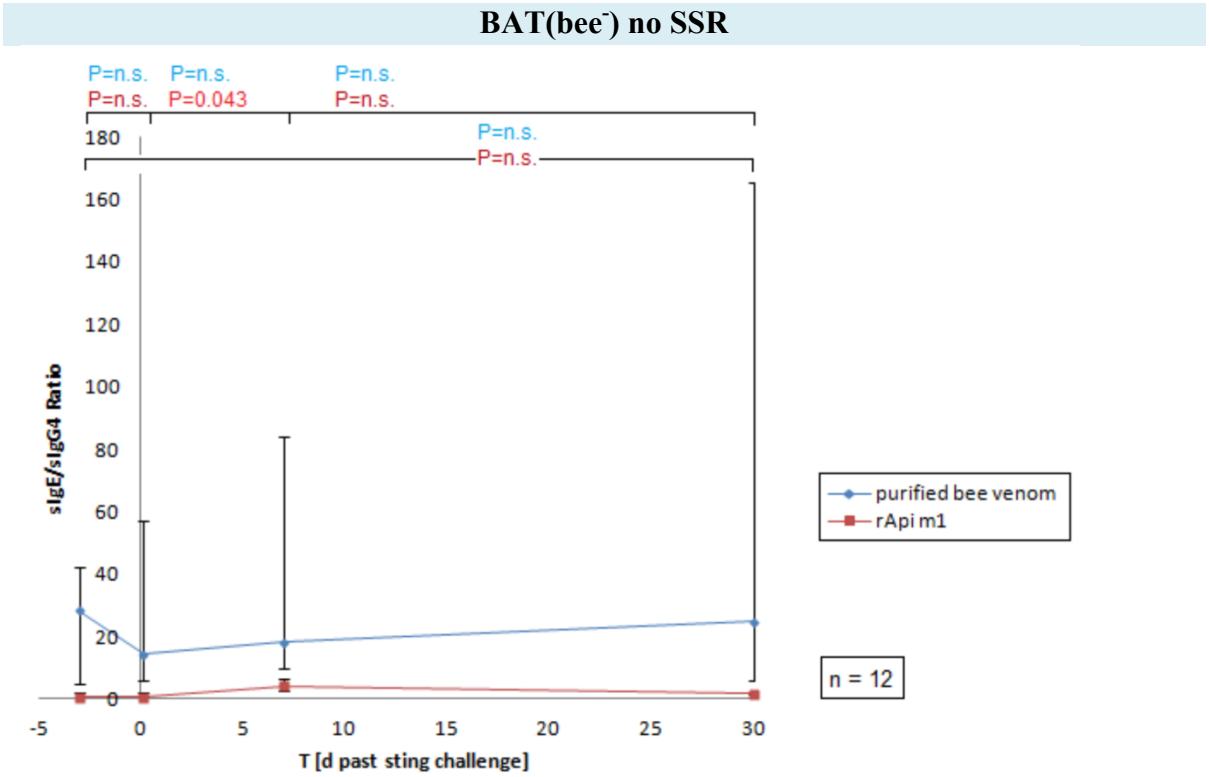
sIgG4 levels of the "BAT(bee$^-$) SSR" patient developed as follows: Over the first 3 hours after the sting, where the patient experienced a grade I-systemic reaction, sIgG4 levels against bee venom and rApi m 1 of seemed unchanged. 7 and 30 days after the sting these sIgG4 levels had increased markedly when compared to levels of patients who did not react systemically to the challenge (figure 19 and 20). Of the "BAT(bee$^-$) no SSR" study group the median sIgG4 level against bee venom displayed an overall increase from 0.14 kU/l to 1.41 kU/l. In contrast, the "BAT(bee$^-$) SSR" patient showed sIgG4 levels that increased from 0.19 and 0.17 kU/l to 5.05 and 6.3 kU/l against bee venom and rApi m 1, respectively. It seems that in the patient experiencing a systemic reaction production of sIgG4 was particularly triggered.

For the "BAT(wasp$^-$) no SSR" sIgG4 levels developed differently: Over the course of the study sIgG4 levels against wasp venom slightly decreased (from 0.15 kU/l to 0.09 kU/l), while that against rVes v 1 and rVes v 5 remained unchanged (figure 24). Also sIgG4 against bee venom and rApi m 1 decreased (figure 21). No statement about the significant relevance of this development can be made.

4.6.3 Time course of the sIgE/sIgG4 ratio

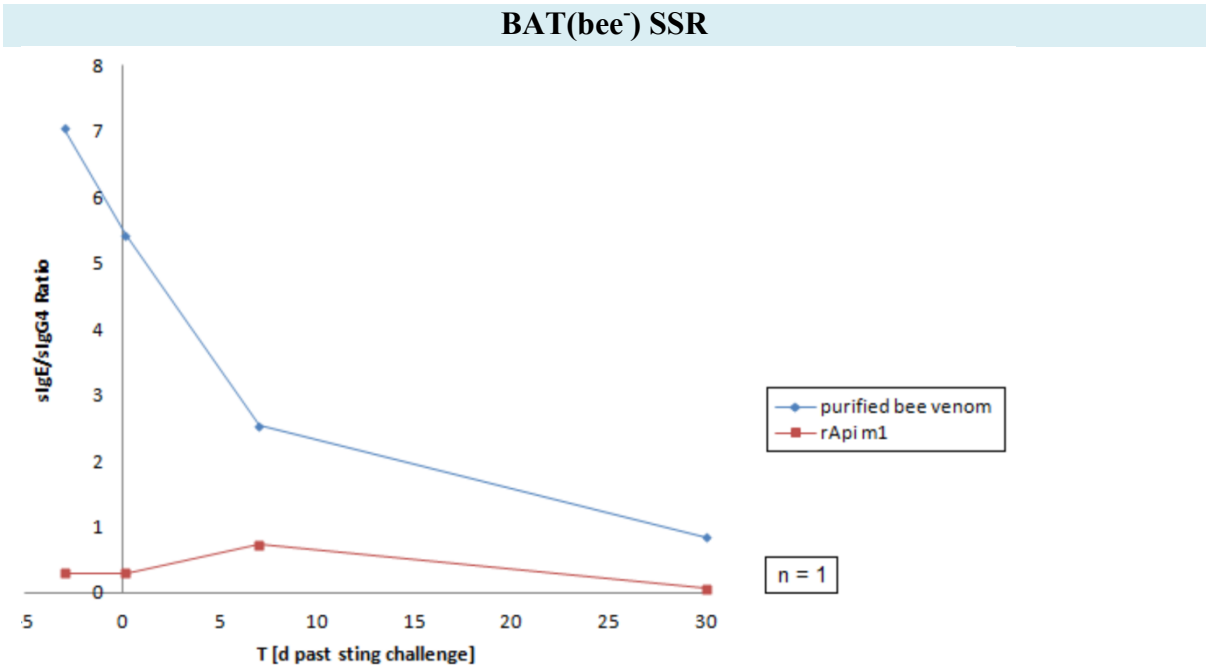
4.6.3.1 sIgE/sIgG4 ratio: bee venom and rApi m 1

Figure 25: "BAT(bee$^-$) no SSR" study group: Time course of the sIgE/sIgG4 ratios for bee venom and rApi m 1



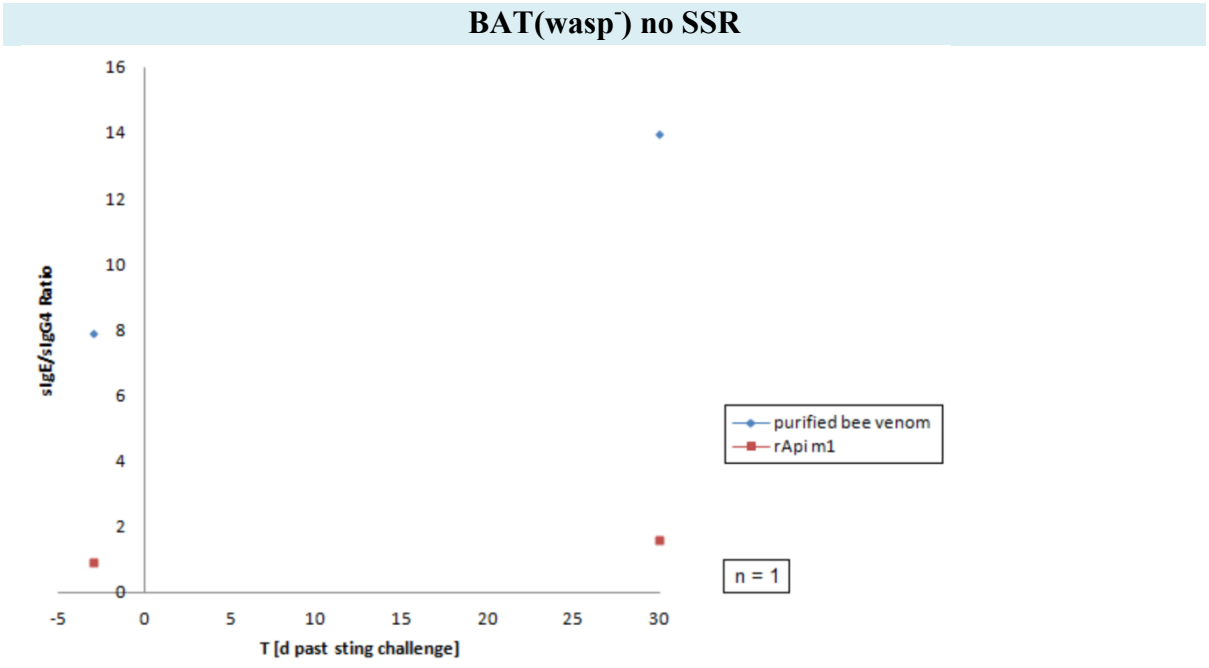
The "BAT(bee$^-$) no SSR" group represents all study individuals for whom bees were detected as BAT negative insect and who did not show systemic reactions when challenged with the sting of a bee. The study group comprised 12 patients. T = 0 indicates the point of sting challenge with a bee. The sIgE/sIgG4 ratios for bee venom and rApi m 1 were analyzed prior to, 3 hours, 7 and 30 days after the challenge.

Figure 26: "BAT(bee⁻) SSR" study individual: Time course of the sIgE/sIgG4 ratios for bee venom and rApi m 1



One patient for whom bees were determined as BAT negative insect showed a systemic anaphylactic reaction in the bee sting challenge, referred to as "BAT(bee-) SSR". T = 0 indicates the point of sting challenge with a bee. The sIgE/sIgG4 ratios for bee venom and rApi m 1 were analyzed prior to, 3 hours, 7 and 30 days after the challenge.

Figure 27: "BAT(wasp⁻) no SSR" study individual: Time course of the sIgE/sIgG4 ratios for bee venom and rApi m 1

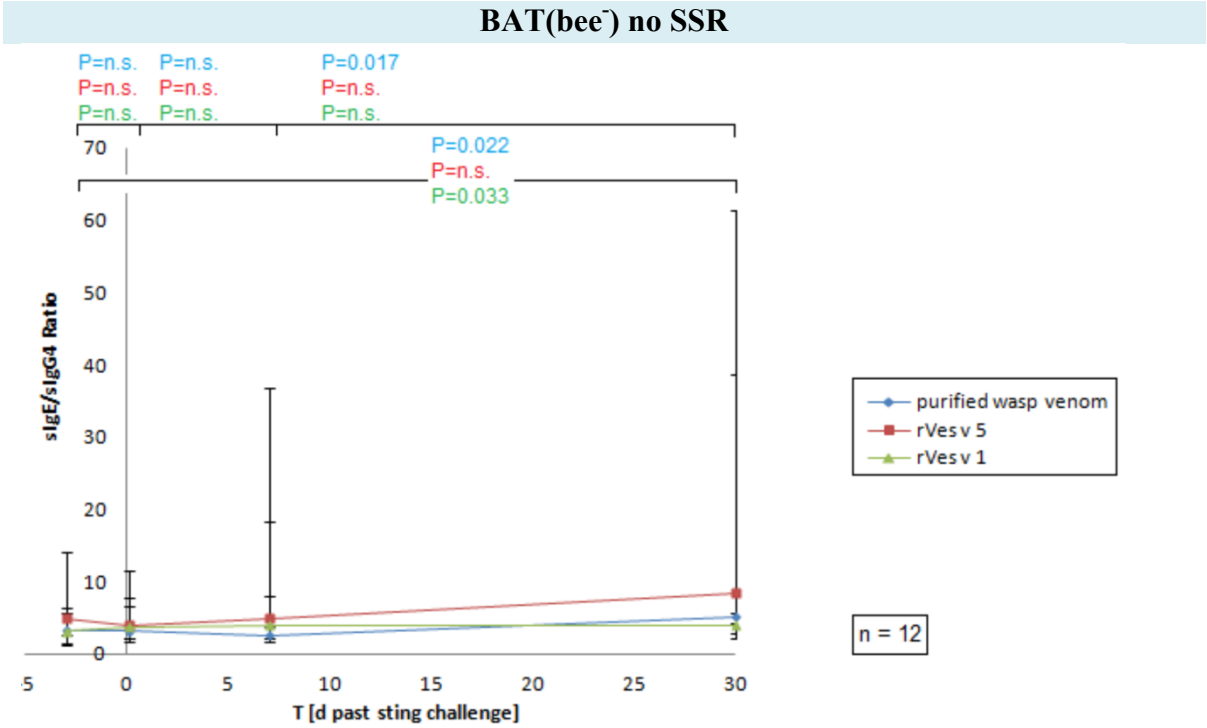


For one patient wasps were determined as BAT negative insect; when exposed to the sting of a wasp no systemic reaction was triggered, referred to as "BAT(wasp-) no SSR". T = 0 indicates the point of sting

challenge with a wasp. The sIgE/sIgG4 ratios for bee venom and rApi m 1 were analyzed prior to and 30 days after the challenge.

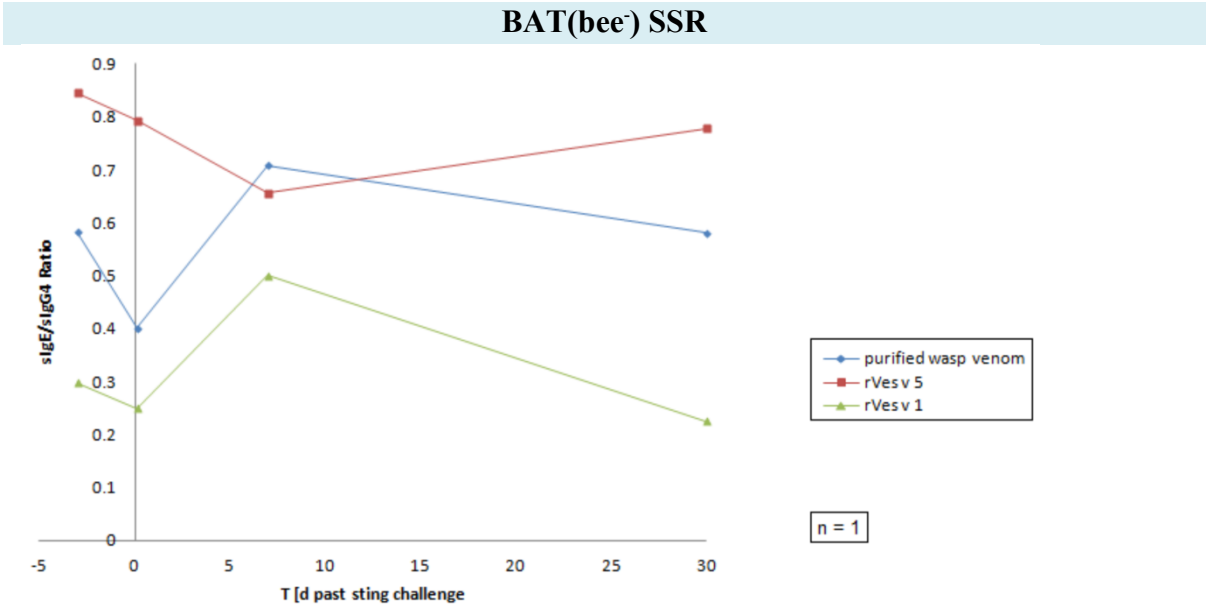
4.6.3.2 sIgE/sIgG4 ratio: wasp venom, rVes v 1 and r Ves v 5

Figure 28: "BAT(bee⁻) no SSR" study group: Time course of the sIgE/sIgG4 ratios for wasp venom, rVes v 1 and rVes v 5



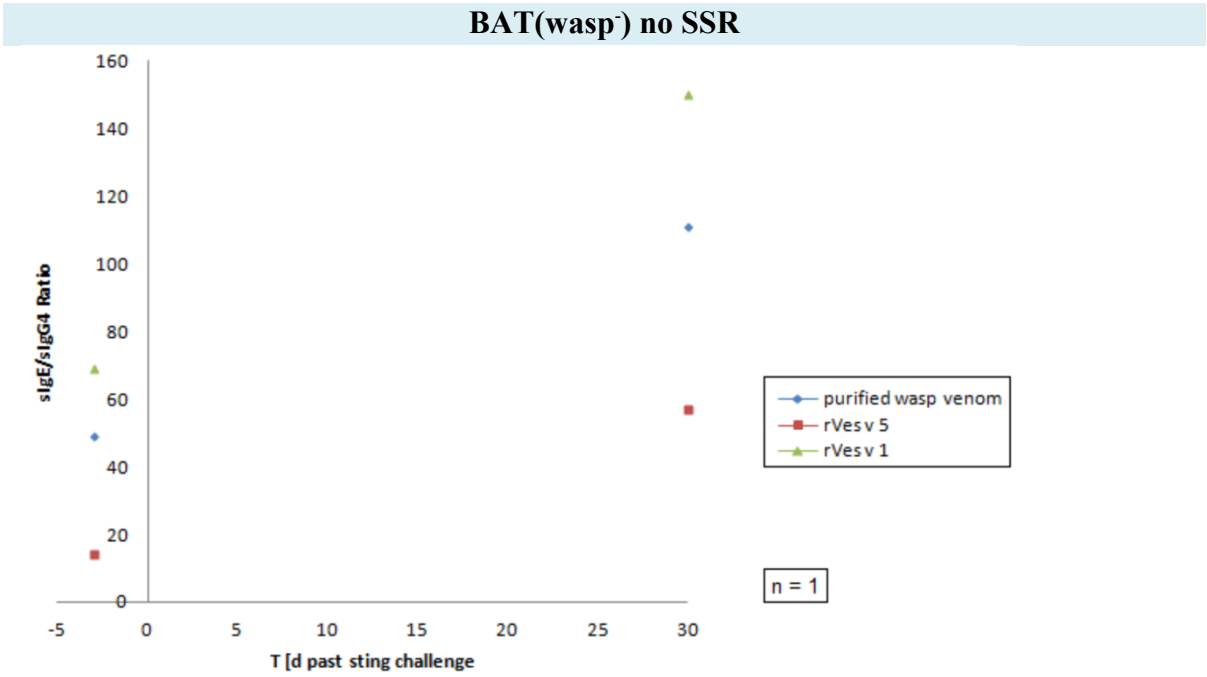
The "BAT(bee⁻) no SSR" group represents all study individuals for whom bees were detected as BAT negative insect and who did not show systemic reactions when challenged with the sting of a bee. The study group comprised 12 patients. T = 0 indicates the point of sting challenge with a bee. The sIgE/sIgG4 ratios for wasp venom, rVes v 1 and rVes v 5 were analyzed prior to, 3 hours, 7 and 30 days after the challenge.

Figure 29: "BAT(bee$^-$) SSR" study individual: Time course of the sIgE/sIgG4 ratios for wasp venom, rVes v 1 and rVes v 5



One patient for whom bees were determined as BAT negative insect showed a systemic anaphylactic reaction in the bee sting challenge, referred to as "BAT(bee-) SSR". T = 0 indicates the point of sting challenge with a bee. The sIgE/sIgG4 ratios for wasp venom, rVes v 1 and rVes v 5 were analyzed prior to, 3 hours, 7 and 30 days after the challenge.

Figure 30: "BAT(wasp$^-$) no SSR" study individual: Time course of the sIgE/sIgG4 ratios for wasp venom, rVes v 1 and rVes v 5



For one patient wasps were determined as BAT negative insect; when exposed to the sting of a wasp no systemic reaction was triggered, referred to as "BAT(wasp$^-$) no SSR". T = 0 indicates the point of sting

challenge with a wasp. The sIgE/sIgG4 ratios for wasp venom, rVes v 1 and rVes v 5 were analyzed prior to and 30 days after the challenge.

While sIgE antibodies determine sensitization to allergens, sIgG4 antibodies are thought to have some blocking effects on allergic reactions. (39) The ratio of sIgE to sIgG4 is thus of interest, in particular when comparing individuals who did not react to the sting challenge to the patient who reacted systemically to it.

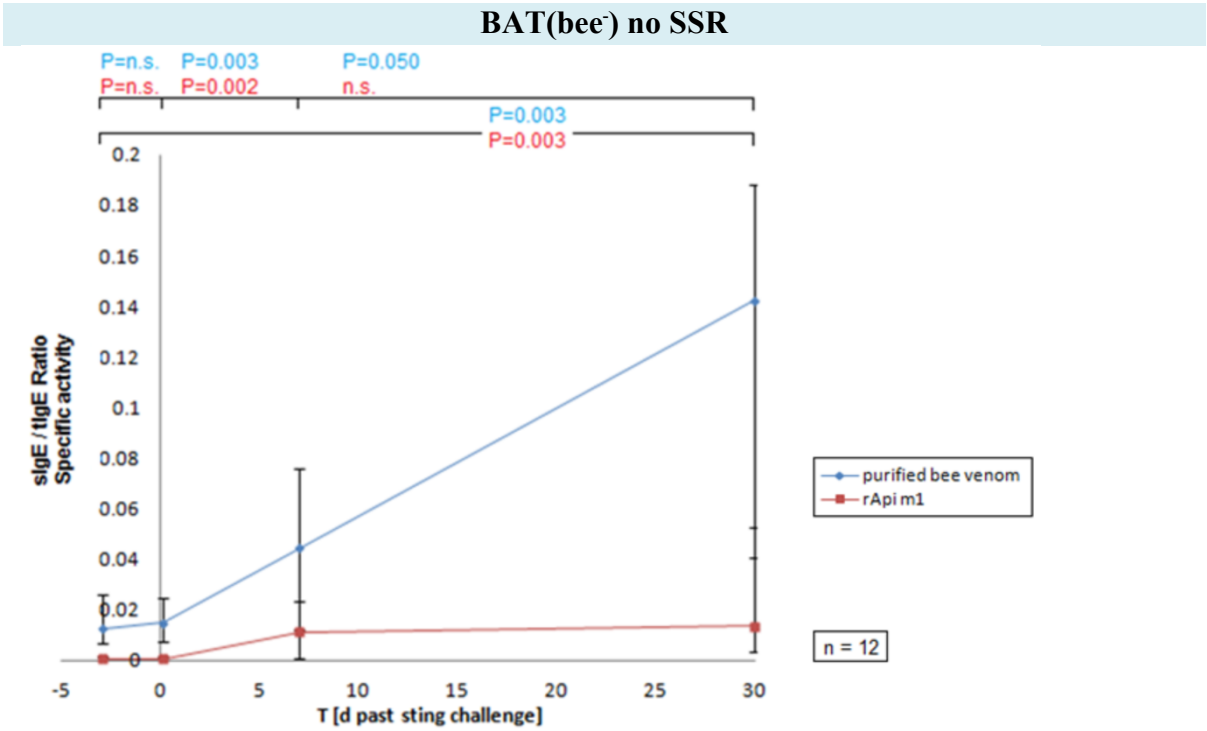
The "BAT(bee$^-$) no SSR" study group showed a significant increase in this ratio for rApi m 1 for the period between 3 hours and 7 days after the sting. Over the entire course of the study no significant changes of the sIgE:sIgG4 ratio for bee venom or rApi m 1 could be observed. (figure 25) The ratios among this group were however widely distributed and thus no significant results could be deduced from this data. Interestingly, a significant increase of this ratio for wasp venom and rVes v 1 was found. (figure 28)

In comparison, for the "BAT(bee$^-$) SSR" patient the sIgE:sIgG4 ratio for bee venom showed a clear decrease reflecting the elevation of sIgG4 over the study course. A decrease in this ratio for rApi m 1 could also be seen. It was however not as prominent as that for bee venom. (figure 26) Due to the wide data distribution among the "BAT(bee$^-$) no SSR" group, a comparison with the development in the "BAT(bee$^-$) SSR" patient was not possible.

4.6.4 Time course of the IgE - specific activity

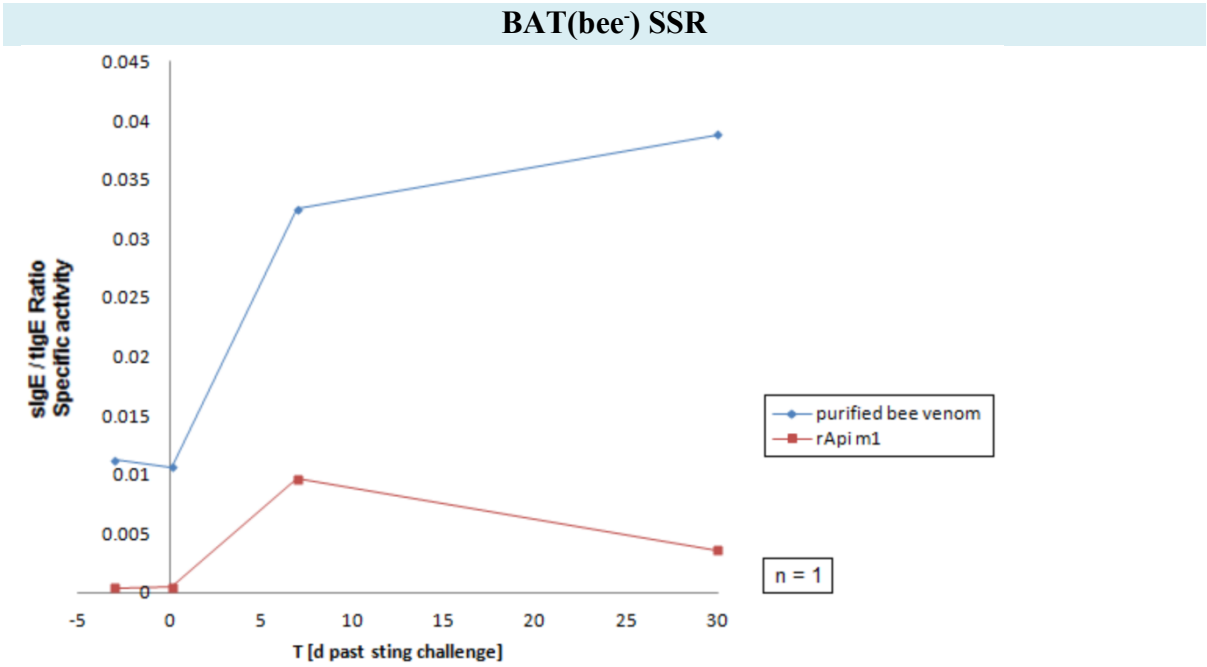
4.6.4.1 IgE – specific activity: bee venom and rApi m 1

Figure 31: "BAT(bee$^-$) no SSR" study group: Time course of the IgE – specific activities (Ratio: sIgE/tIgE) for bee venom and rApi m 1



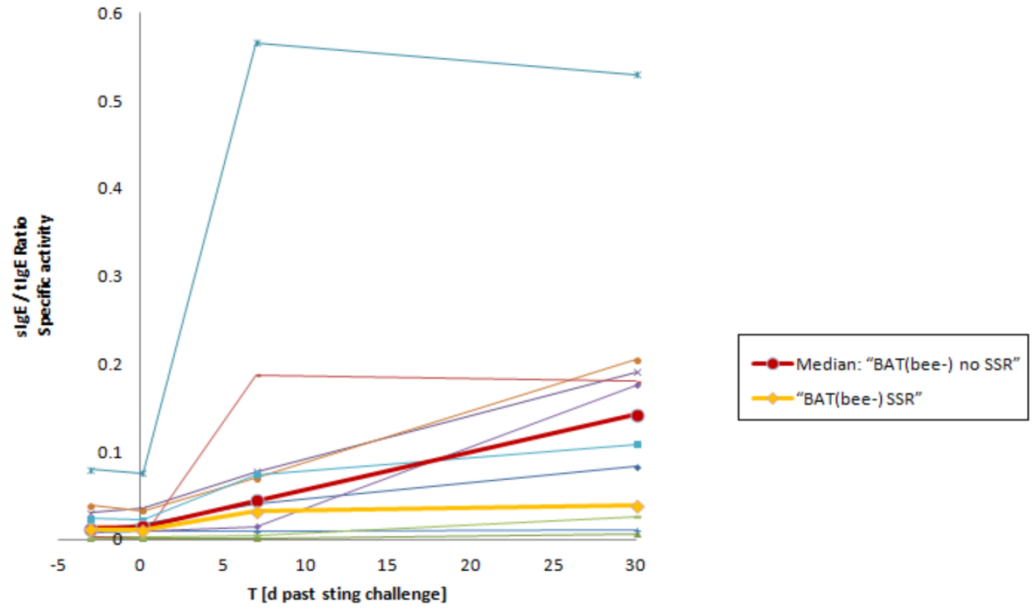
The "BAT(bee$^-$) no SSR" group represents all study individuals for whom bees were detected as BAT negative insect and who did not show systemic reactions when challenged with the sting of a bee. The study group comprised 12 patients. T = 0 indicates the point of sting challenge with a bee. The IgE – specific activities for bee venom and rApi m 1 were analyzed prior to, 3 hours, 7 and 30 days after the challenge.

Figure 32: "BAT(bee⁻) SSR" study individual: Time course of the IgE – specific activities (Ratio: sIgE/tIgE) for bee venom and rApi m 1



One patient for whom bees were determined as BAT negative insect showed a systemic anaphylactic reaction in the bee sting challenge, referred to as "BAT(bee⁻) SSR". T = 0 indicates the point of sting challenge with a bee. The IgE – specific activities for bee venom and rApi m 1 were analyzed prior to, 3 hours, 7 and 30 days after the challenge.

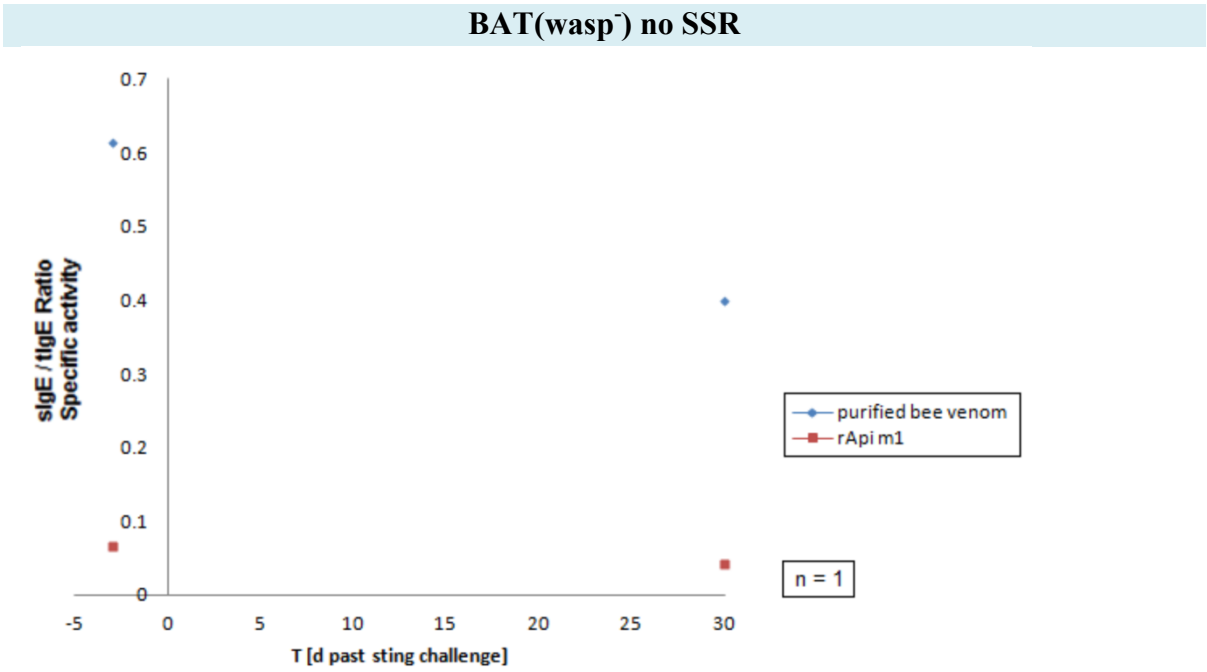
Figure 33: Time course of the IgE- specific activity for bee venom: "BAT(bee⁻) no SSR" vs. "BAT(bee⁻) SSR"



Comparison of the time course of the IgE-specific activity for purified bee venom of all study patients that were challenged with the sting of a bee. T = 0 represents the point of sting challenge. The median

(red) of the "BAT(bee⁻) no SSR" group and the "BAT(bee⁻) SSR" patient (yellow) are highlighted, where "BAT(bee⁻) SSR" represents the only study individual that experienced a systemic anaphylactic reaction grade I to the bee sting challenge.

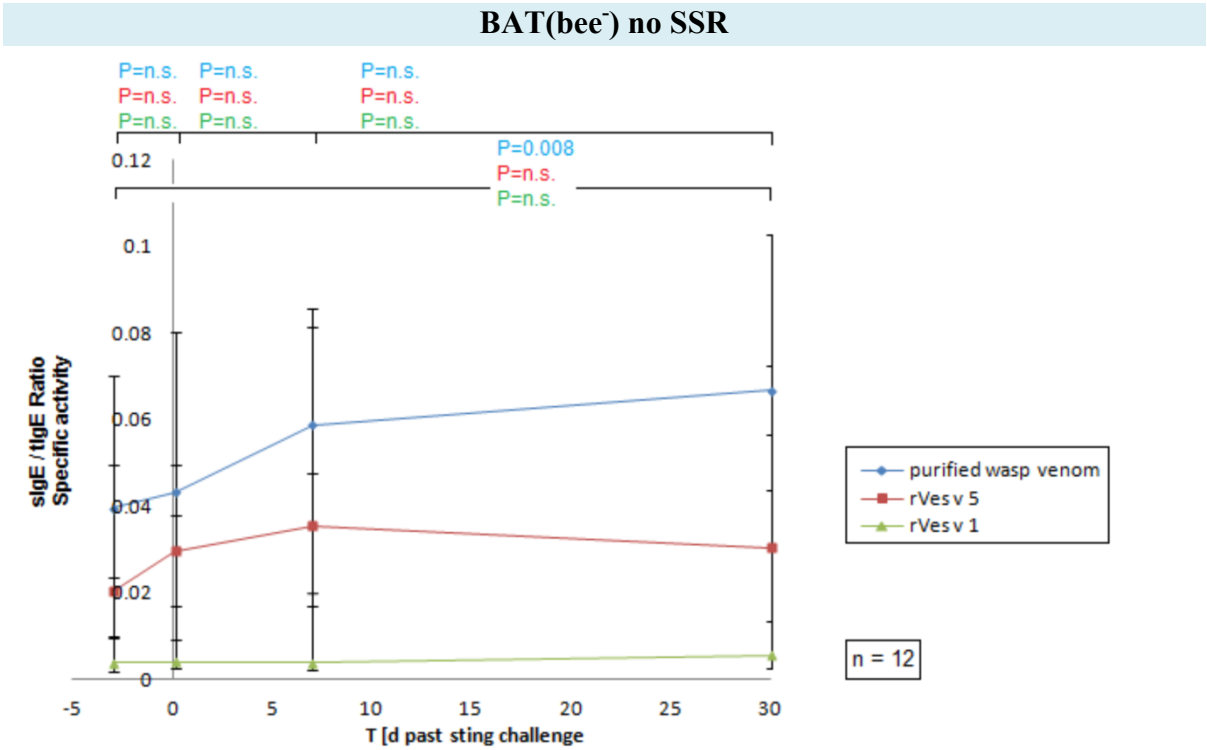
Figure 34: "BAT(wasp⁻) no SSR" study individual: Time course of the IgE – specific activities (Ratio: sIgE/tIgE) for bee venom and rApi m 1



For one patient wasps were determined as BAT negative insect; when exposed to the sting of a wasp no systemic reaction was triggered, referred to as "BAT(wasp⁻) no SSR". T = 0 indicates the point of sting challenge with a wasp. The IgE – specific activities for bee venom and rApi m 1 were analyzed prior to and 30 days after the challenge.

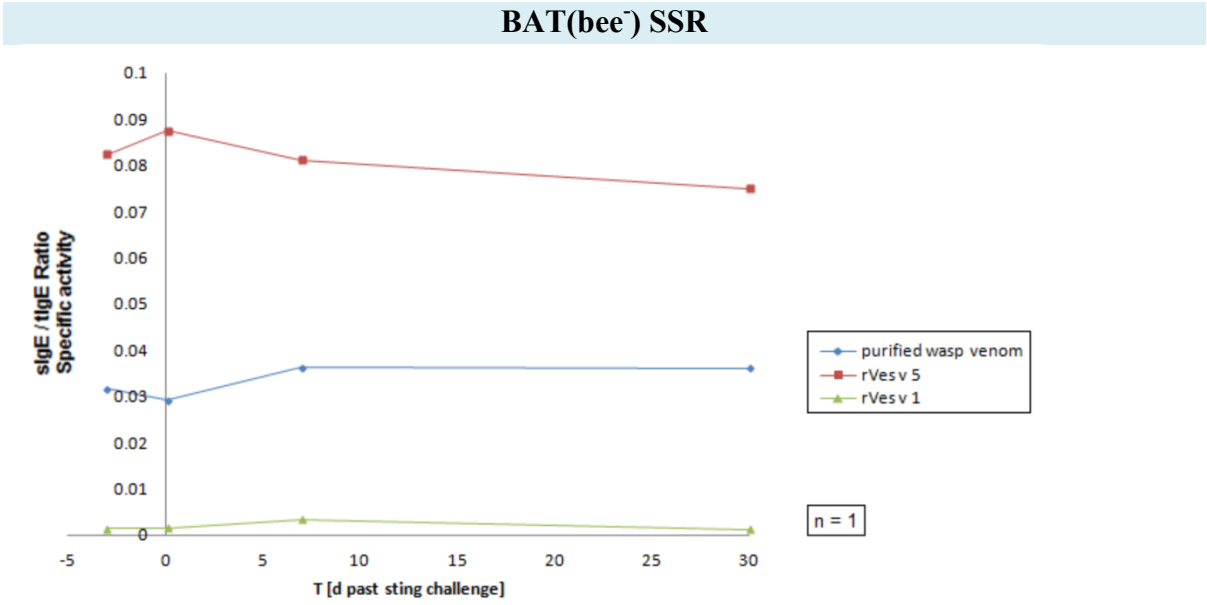
4.6.4.2 IgE – specific activity: wasp venom, rVes v 1 and rVes v 5

Figure 35: "BAT(bee⁻) no SSR" study group: Time course of the IgE – specific activities (Ratio: sIgE/tIgE) for wasp venom, rVes v 1 and rVes v 5



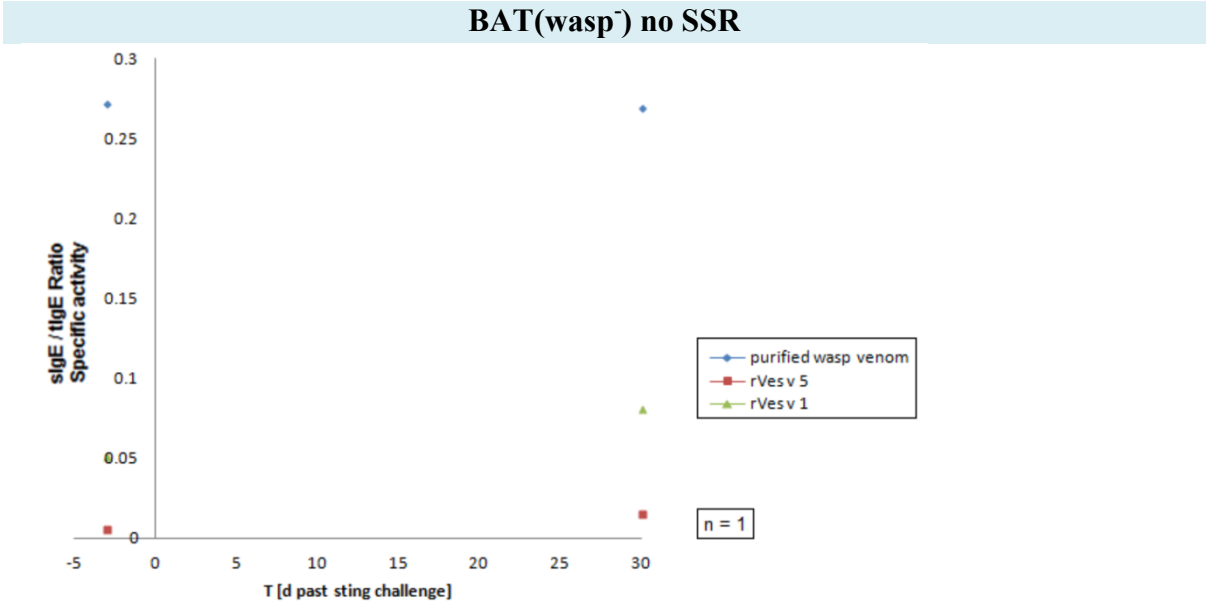
The "BAT(bee⁻) no SSR" group represents all study individuals for whom bees were detected as BAT negative insect and who did not show systemic reactions when challenged with the sting of a bee. The study group comprised 12 patients. T = 0 indicates the point of sting challenge with a bee. The IgE – specific activities for waps venom, rVes v 1 and rVes v 5 were analyzed prior to, 3 hours, 7 and 30 days after the challenge.

Figure 36: "BAT(bee⁻) SSR" study individual: Time course of the IgE – specific activities (Ratio: sIgE/tIgE) for wasp venom, rVes v 1 and rVes v 5



One patient for whom bees were determined as BAT negative insect showed a systemic anaphylactic reaction in the bee sting challenge, referred to as "BAT(bee-) SSR". T = 0 indicates the point of sting challenge with a bee. The IgE – specific activities for wasp venom, rVes v 1 and rVes v 5 were analyzed prior to, 3 hours, 7 and 30 days after the challenge.

Figure 37: "BAT(wasp⁻) no SSR" study individual: Time course of the IgE – specific activities (Ratio: sIgE/tIgE) for wasp venom, rVes v 1 and rVes v 5



For one patient wasps were determined as BAT negative insect; when exposed to the sting of a wasp no systemic reaction was triggered, referred to as "BAT(wasp⁻) no SSR". T = 0 indicates the point of sting challenge with a wasp. The IgE – specific activities for wasp venom, rVes v 1 and rVes v 5 were analyzed prior to and 30 days after the challenge.

The IgE-specific activity is the ratio of sIgE:tIgE and was suggested as marker for IgE antibody responses. (38) The time course of the IgE-specific activity in the "BAT(bee$^-$) no SSR" group is similar to that of sIgE levels. An overall increase in IgE-specific activities for bee venom and rApi m 1 was observed (figure 31). Interestingly, also the IgE-specific activity for wasp venom rose significantly (figure 35).

Also the time course of the IgE-specific activity in the "BAT(bee$^-$) SSR" patient reflects that of sIgE levels against bee venom and rApi m 1. For bee venom a slight drop was found 3 hours after the challenge followed by a noticeable rise over the remaining study course. (figure 32) When compared to patients of the BAT(bee$^-$) no SSR" group this increase was not as prominent as for most of them (figure 33). No statement about the significance of this development can be made.

5 Discussion

Double-positive results in skin tests and sIgE quantification pose a major problem for correct choice of venom for specific immunotherapy. The BAT has been generally recognized as an additional and reliable tool in the diagnosis of Hymenoptera venom allergy. (50) Several studies have confirmed the usefulness of the BAT in identifying the clinically relevant insect venom (30,37,57). Also in cases of ambiguous or contradictory results in standard tests, the BAT has shown to be a useful additional method in determining the culprit insect. (27,49).

In contrast to these studies, the focus of the current study was put on the ability of the BAT to identify clinically irrelevant insect venoms, in particular when confronted with double-positivity in standard tests. Furthermore we aimed to demonstrate that a negative result in BAT has a high negative predictive value.

For that purpose 14 patients with double-sensitization in IDT and CAP were analyzed with two different protocols of CD63-based BAT. Negative results in BAT were confirmed by sting challenge tests.

The current study clearly demonstrates the ability of the BAT to determine clinically irrelevant insect venoms. For both BAT protocols high negative predictive values were determined. A negative predictive value of 87.5% for the BAT Graz and 92.9% for the BAT Bühlmann were found. A combination of both BAT protocols yielded a negative predictive value of 92.9%.

For the BAT Bühlmann Scherer K. et al. (2008) reported similar findings. In 178 study subjects with histories of systemic reactions to wasp or bee stings negative predictive values for bee and wasp venom were determined 89.5% and 86.4%, respectively (53). In the study approach of Scherer et al. clinical diagnosis was based on history alone. Patients however are often not able to correctly identify the culprit insect of a systemic sting reaction. In the current study, sting challenge tests with the BAT negative insects were performed to confirm negative BAT results. The approach of the current study is clearly better suited and more accurate in determining negative predictive values.

In this study the negative predictive value of that BAT Graz protocol was examined for the first time, so no comparable study data are available.

The current study also clearly confirms the BAT as valuable, additional diagnostic tool in Hymenoptera venom allergy, in particular when confronted with double-sensitization in IDT and CAP. In a study involving 117 patients with a history of systemic sting reactions Sturm G.J. et al. (2011) demonstrated that with the BAT considerably less double-positive

results are obtained than with CAP and also IDT (45). In the current study, double-sensitization was found in 43% of patients analyzed with the BAT Graz. The BAT Bühlmann showed exceptional performance in this area, where mono-sensitization was determined for all analyzed study subjects.
As a relative small population of patients was analyzed, these findings should be confirmed in studies with larger subject numbers.

In the current study one patient reacted systemically (grade I) to the sting of a bee that was previously determined to be clinically irrelevant by both BAT protocols. No exceptional testing results in IDT and CAP compared to other subjects could be found. Concerning CRD, sensitization to rApi m 1 was negative.
Sturm G.J. et al. (2011) and Korosec et al. (2011) reported a low sensitivity of sIgE quantification against rApi m 1 (47,58), possibly explaining the patient's reaction despite negative sensitization to the major bee allergen. Nevertheless, this does not explain why bee allergy failed to be detected by both BAT protocols.
In sensitized patients however allergies can manifest with every new exposure to allergens. It may thus be that the sting challenge with a bee has caused manifestation of a bee allergy in that patient.

The observed time courses of sIgE and sIgG4 levels to allergens of the challenging insects were as expected. The sting of the insects stimulated sIgE and sIgG4 production against the respective allergens (9,11).
Interestingly, in subjects challenged with a bee who did not show systemic reactions to it, a significant increase in sIgE against wasp venom was triggered. Part of this increase may be accounted to CCD-based cross-reactivity, as no such rise of sIgE against rVes v 5 was observed. rVes v 1 and rVes v 5 are known to be non-glycosylated allergens unaffected by CCD-based cross-reactivity (44). However, sIgE levels against rVes v 1 showed a significant increase 30 days after the challenge. The cause of this increase has to be investigated in further studies.
For the same subjects a decrease in sIgG4 against bee venom was observed 3 hours after the sting. This could suggest that blocking sIgG4 were used up during the immediate reaction phase and later increasingly rebuild. In contrast, in the patient experiencing a grade-I systemic reaction no such decrease was seen. Moreover, it seems that for this patient production of sIgG4 was particularly triggered. To determine if this difference in

sIgG4 development is significant, studies involving a larger population of subjects need to be performed.

To conclude, it can be said that the BAT is a valuable additional tool in diagnosis of Hymenoptera venom allergy. Its usefulness is not restricted to determine the culprit insect, but with high negative predictive values the BAT has the advantage of being able to identify also clinically irrelevant sensitization.

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