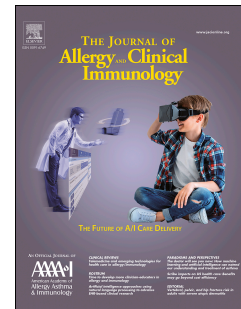


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Glycosylation enhances allergenic activity of major bee venom allergen Api m 1 by adding IgE epitopes

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Key words: insect venom allergy, Api m 1, allergen, glycosylation, allergenic activity

Abbreviations:

n natural

r recombinant

CCD cross-reactive carbohydrate determinants

kDa kilo Dalton

ELISA enzyme linked immunosorbent assay

Galactose- α -1,3-galactose α -GAL

Aleuria aurantia Lectin AAL

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Author Contributions:

PG, IM: Performed experiments, analysed data, wrote manuscript, read manuscript; UBS, MZ, PK: provided samples and clinical data, analysed data, read manuscript; RV, IM: designed and supervised experiments, analysed data, wrote and read manuscript.

Conflict of Interest Statement:

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Capsule Summary

Glycosylation is not required for structural fold and function of bee venom allergen Api m 1 but enhances allergenic activity by adding IgE epitopes, which is important for diagnosis and therapy of bee venom allergy.

To the Editor:

Phospholipase A₂ (Api m 1), the major bee venom allergen is a glycoprotein, which can bind allergic patients' IgE antibodies via protein and/or carbohydrate epitopes.¹ It has been suggested that the protein epitopes of Api m 1 are conformation-dependant² but recently also IgE-reactive sequential epitopes have been described.³ Carbohydrates present on allergens from plants, insects, mites, moulds and parasites have been considered as highly cross-reactive epitopes (i.e., cross-reactive carbohydrate determinants: CCD) with low capacity to induce basophil or mast cell degranulation and hence with low allergenic activity.⁴ Accordingly, the presence of CCDs on allergens may cause misleading IgE test results. One important example is that grass pollen allergic patients with IgE reactivity against Phl p 4-derived CCDs showed IgE reactivity to natural, CCD-containing Api m 1

64 although they were not sensitized against bee venom.¹ In order to avoid such false IgE test results
 65 non-glycosylated Api m 1 has been introduced for serological diagnosis of bee venom sensitivity.⁵
 66 Conversely, IgE antibodies to the carbohydrate Galactose- α -1,3-galactose (α -GAL) can trigger
 67 allergic reactions.⁶
 68 The influence of glycosylation and enzymatic activity on allergenic activity of the major bee venom
 69 allergen, Api m 1 is a controversial issue. Certain studies suggest similar allergenic activity of
 70 natural glycosylated and recombinant non-glycosylated Api m 1², whereas enzymatic activity seems
 71 to be important for allergenic activity.⁷ In order to investigate the role of bee-derived glycosylation
 72 on structural fold, enzymatic activity and allergenic activity of Api m 1, we compared purified
 73 natural Api m 1 with non-glycosylated recombinant Api m 1 expressed in insect cells. Recombinant
 74 non-glycosylated Api m 1 with a mutated N-glycosylation site was expressed in *Spodoptera*
 75 *frugiperda* (Sf9) insect cells (Methods in this article's Online Repository at www.jacionline.org) and
 76 natural glycosylated Api m 1 was obtained from Sigma-Aldrich, Vienna, Austria. The Coomassie-
 77 stained SDS-PAGE and corresponding Westernblot showed that rApi m 1 was pure and migrated as
 78 a 15 kDa band, whereas nApi m 1 appeared in the form of two bands, one prominent at 16-17 kDa,
 79 one at 14 kDa. According to staining with α -(1,3)-fucose-specific *Aleuria aurantia* Lectin (AAL),
 80 the 16-17 kDa nApi m 1 band was glycosylated, whereas rApi m 1 lacked glycosylation (see Fig
 81 E1A, B and methods in this article's Online Repository at www.jacionline.org). Thus, Figure E1
 82 shows that nApi m 1 consists of a glycosylated and non-glycosylated fraction. The thin band at 14
 83 kDa corresponds to the non-glycosylated and the prominent band at 16-17 kDa represents the
 84 glycosylated version. rApi m 1, which is not glycosylated, gives a single band migrating at 15 kDa.
 85 nApi m 1 as well as rApi m 1 showed specific IgE-reactivity by IgE immunoblotting (Figure E1C),
 86 however IgE levels specific for nApi m 1 were 1.2 to 2.71-fold higher in 8 out of 12 bee venom
 87 allergic patients (Table E1 and methods in this article's Online Repository at www.jacionline.org).
 88 By contrast, there was a good correlation between the rApi m -specific IgE levels determined by

ImmunoCAP and ELISA but differences were noted for certain sera (Figure E2, Table E1). For both assays (ImmunoCAP and ELISA) rApi m 1 is expressed in the insect cell line SF9 and lacks the N-linked glycosylation site.⁵ Explanation for the observed discrepancies may be that the ImmunoCAP contains large amounts of antigen immobilized to its surface, whereas much lower amounts of antigen are coated to the ELISA plates, which may allow IgG antibodies to compete with IgE for binding in the ELISA resulting in lower IgE signals. Finally, ImmunoCAPs may bind CCD-specific IgE in non-specific manner, which could pretend higher IgE levels in the ImmunoCAP measurements for sera containing CCD-specific IgE.⁸

Next we investigated whether there are differences regarding fold and functional properties of the natural and recombinant Api m 1 protein. However, the analysis of nApi m 1 and rApi m 1 by circular dichroism analysis showed that both proteins were folded and no relevant difference regarding secondary structure contents and molar ellipticities could be detected (Fig 1A and methods in this article's Online Repository at www.jacionline.org). Furthermore, nApi m 1 and rApi m 1 occurred as monomeric proteins in physiological buffers as demonstrated by size-exclusion chromatography (Figure E3 and methods in this article's Online Repository at www.jacionline.org). The higher IgE reactivity of nApi m 1 can therefore not be explained by differences in the secondary structure but rather seems to be due to the attached CCDs, because most of the patients were tested positive for CCD-specific IgE (Table E1).

Both proteins, nApi m 1 and rApi m1, but not a control allergen (i.e., birch pollen allergen Bet v 1) showed comparable phospholipase activity during assessment of enzymatic activity in the first 5 minutes of the 15 minute recording period (Fig 1B, methods in this article's Online Repository at www.jacionline.org). During the last 10 minutes of measurement a 20% higher activity of nApi m 1 was found, which may be due to the fact that nApi m 1 and rApi m 1 required different pH conditions (nApi m 1: pH 7.5; rApi m 1: pH 6) for storage that could affect phospholipase activity (Fig 1B). Next, we investigated the allergenic activity of nApi m 1 and non-glycosylated rApi m 1 in

114 titrated basophil activation experiments (Fig 2 and methods in this article's Online Repository at
 115 www.jacionline.org). Four of the five tested patients (patients #6, 3, 9 and 12) (Table E1) showed
 116 stronger basophil activation with nApi m 1 than with rApi m 1, whereas it was similar for patient 4,
 117 who mounted similar levels of specific IgE to nApi m 1 and rApi m 1 (Table E1 in this article's
 118 Online Repository at www.jacionline.org). The stronger basophil activation in the four patients was
 119 associated with higher IgE levels to nApi m 1 than to rApi m 1 (Table E1 in this article's Online
 120 Repository at www.jacionline.org). Interestingly, the difference in allergenic activity for nApi m 1
 121 and rApi m 1 was different from that observed for allergens or allergen derivatives, which differ
 122 regarding IgE reactivity due to differences in structural fold such as for example for folded, insect
 123 cell- versus unfolded *Escherichia coli*-expressed Par j 2.⁹ In fact for allergen molecules, which show
 124 different IgE reactivity due to alterations of their structural fold a shift of the dose response curve can
 125 be observed in basophil activation, whereas nApi m 1 and rApi m 1 showed a comparable dose-
 126 dependency of basophil activation but nApi m 1 induced stronger release of β -hexosaminidase at
 127 each of the concentrations (Fig 2). Such a qualitative difference in basophil activation is usually
 128 observed when basophils contain more allergen-specific IgE (reference E4 in this article's Online
 129 Repository at www.jacionline.org).

130 Our study thus demonstrates that structural fold, enzymatic activity, IgE binding capacity and
 131 allergenic activity of Api m 1 do not depend on glycosylation but glycosylation may increase IgE
 132 reactivity through the additional presence of IgE-reactive CCDs, which in the case of Api m 1
 133 enhances the allergenic activity of the allergen. Our findings are different from what has been
 134 described for other glycosylated allergens. For example, natural Lyc e 2 from tomato bound IgE only
 135 in the glycosylated form and only the glycosylated Lyc e 2 was able to trigger histamine release
 136 (reference E5 in this article's Online Repository at www.jacionline.org). Non-glycosylated house
 137 dust mite allergen Der p 1 had enzymatic activity, was folded correctly and induced histamine
 138 release in basophil activation test but no evidence was provided that glycosylation enhances

allergenic activity as we found for Api m 1 (reference E6 in this article's Online Repository at www.jacionline.org). By contrast, Bla g 2, a German cockroach allergen, seems to be a similar example as Api m 1, because it was shown that removal of sugars resulted in a reduction of IgE binding and allergenic activity as demonstrated in histamine release experiments with human basophils (references E7- E9 in this article's Online Repository at www.jacionline.org).

According to available data one can envisage different possibilities how glycosylation may affect the allergenic activity of an allergen. Carbohydrate epitopes may mask protein-based IgE epitopes as has been shown for grass pollen allergen Phl p 4 (reference E10 in this article's Online Repository at www.jacionline.org). Carbohydrates may improve the fold of an allergen and hence stabilize conformational IgE epitopes, which would enhance allergenic activity (reference E11 in this article's Online Repository at www.jacionline.org). Finally, carbohydrates may add carbohydrate IgE epitopes in addition to protein-based IgE epitopes. In fact Api m 1 seems to be a first example for the latter case, because glycosylation adds carbohydrate-specific IgE epitopes to the peptide IgE epitopes on an otherwise folded, IgE-reactive, allergenic and enzymatically active, non-glycosylated protein to increase its allergenic activity. This mechanism may be also operative for Bla g 2, where the carbohydrates seem to contribute to IgE antibody binding to the protein within the same epitope (E7, E9 in this article's Online Repository at www.jacionline.org).

This result has important implications for the use of Api m 1 for diagnostic purposes and presumably for therapeutic purposes as well. nApi m 1 seems to be more effective in inducing basophil activation and hence may be more potent in effector cell-based assay and in *in vivo* provocation tests, whereas folded non-glycosylated insect cell expressed rApi m 1 may increase specificity of *in vitro* IgE testing because it avoids misleading test results due to presence of CCDs.

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Figure legends

FIG 1. Circular dichroism analysis of natural and recombinant Api m 1. **(A)** Scans showing molecular ellipticities (y-axes) at given wavelengths (x-axes) as shown for nApi m 1 (Api m 1 nat, left) and rApi m 1 (Api m 1 rec, right). **(B)** Phospholipase A₂ activity of nApi m 1 and rApi m 1. Results are expressed as time-dependent increases of absorbance (mOD405nm/min). Mean values of duplicate measurements are shown.

FIG 2. Allergenic activity of nApi m 1 and rApi m 1. RBL cells were loaded with serum IgE from bee venom allergic patients (Table E1: patients #3, 4, 6, 9, 12) and incubated with different concentrations of nApi m 1 or rApi m 1 (100, 10, 1, 0.1, 0.01 ng/mL) (x-axes) or buffer alone. β -hexosaminidase releases are displayed as mean percentages of duplicate determinations displaying the ranges of variance of total β -hexosaminidase release minus buffer on the y-axes.

TABLE E1. Clinical and serological characterization of bee venom allergic patients.

Patients ID	Total serum IgE (kU/L) ^{a)}	Bee venom IgE (kUA/L) ^{b)}	rApi m 1 (kUA/L) ^{c)}	nApi m 1 O.D. ^{d)e)} Range of individual measurements (lowest – highest)	nApi m 1 O.D. ^{d)e)} Mean	rApi m 1 O.D. ^{d)e)} Range of individual measurements (lowest – highest)	rApi m 1 O.D. ^{e)f)} Mean	fold increase nApi m 1 to rApi m 1	Mueller Grade ^{g)}	CCD - specific IgE detected with the tested markers ^{h)}
1	496	100	7.51	1.70 - 1.73	1.72	1.37 - 1.41	1.39	1.2	2	no
2	304	8.81	2.37	0.45 - 0.46	0.46	0.46 - 0.47	0.47	0.98	2	no
3	393	100	5.96	1.69 - 1.73	1.71	1.1 - 1.12	1.11	1.5	2	yes
4	n.d.	44.3	4.57	1.32 - 1.34	1.33	1.28 - 1.32	1.30	1.02	4	no
5	474	3.83	16.17	1.89 - 1.88	1.88	1.89 - 1.89	1.89	0.99	2	yes
6	63	1.96	4.43	0.40 - 0.40	0.4	0.22 - 0.23	0.23	1.73	2	yes
7	566	54.1	21.93	1.92 - 1.95	1.94	1.43 - 1.49	1.46	1.3	2	yes
8	385	79.8	2.91	0.48 - 0.52	0.5	0.33 - 0.35	0.34	1.62	2	yes
9	n.d.	10.9	2.63	0.95 - 0.96	0.95	0.34 - 0.35	0.35	2.71	2	yes
10	212	17	7.49	0.89 - 0.93	0.91	0.45 - 0.50	0.48	1.9	3	yes
11	18	7.82	1.59	0.20 - 0.22	0.21	0.2 - 0.2	0.2	1.05	2	no
12	477	n.d	n.d	1.79 - 1.83	1.81	1.30 - 1.33	1.32	1.37	n.d	yes

- a) ImmunoCAP™ (Phadia AB) total IgE
- b) ImmunoCAP™ (Phadia AB), *Apis mellifera* , i1, positive ≥ 0.35 kU/L
- c) IgE-reactivity to non-glycosylated rApi m 1, i208, measured by ImmunoCAP™
- d) IgE-reactivity to glycosylated natural Api m 1 measured by ELISA
- e) All ELISA measurements were performed as duplicates with a variation of less than 6%.
- f) IgE-reactivity to non-glycosylated rApi m 1 measured by ELISA
- g) Clinical severity in Mueller grade
- h) IgE-reactivity to CCDs was determined by recombinant CCD marker HHM2 (see reference 8 this article's Online Repository at www.jacionline.org), CCD-ImmunoCAP and Bromelain-ImmunoCAP.
Abbr.: n.d. not done, O.D. optical density

Fig 1A.

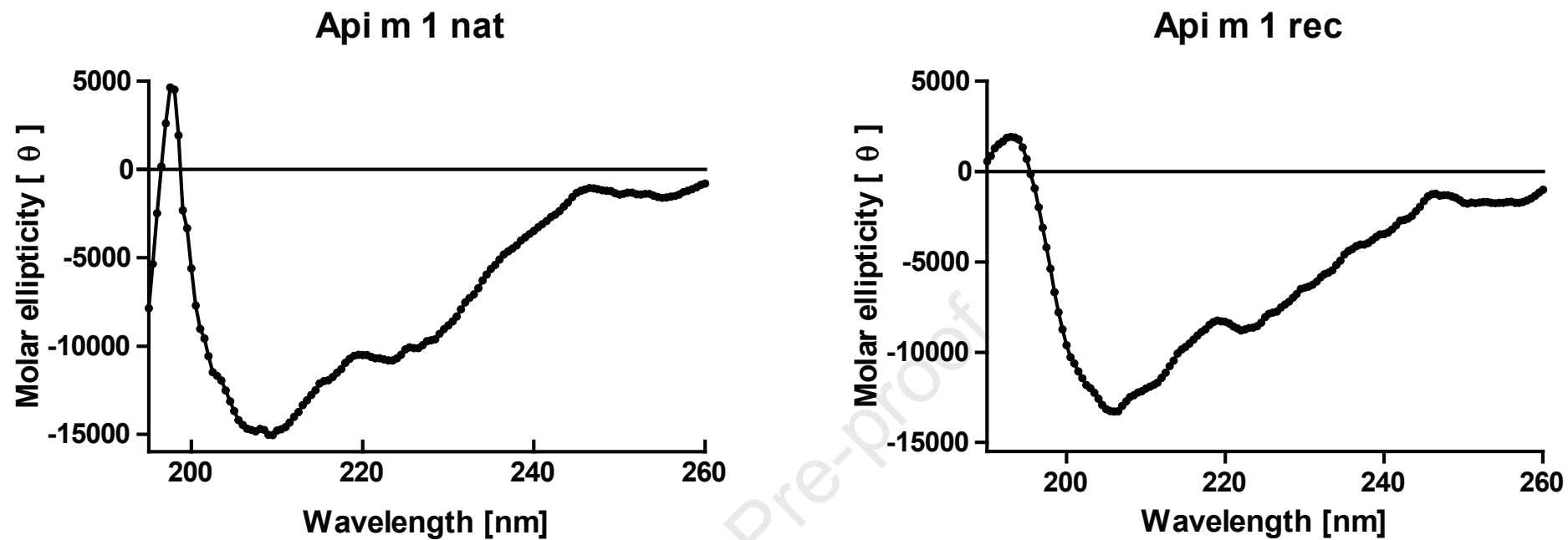


Fig 1B.

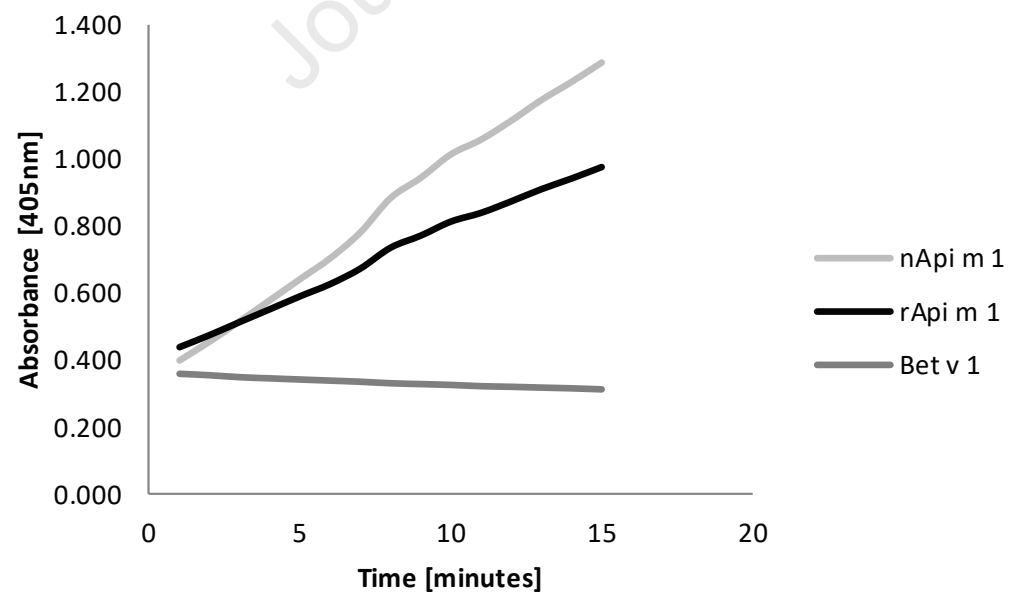


Fig 2.

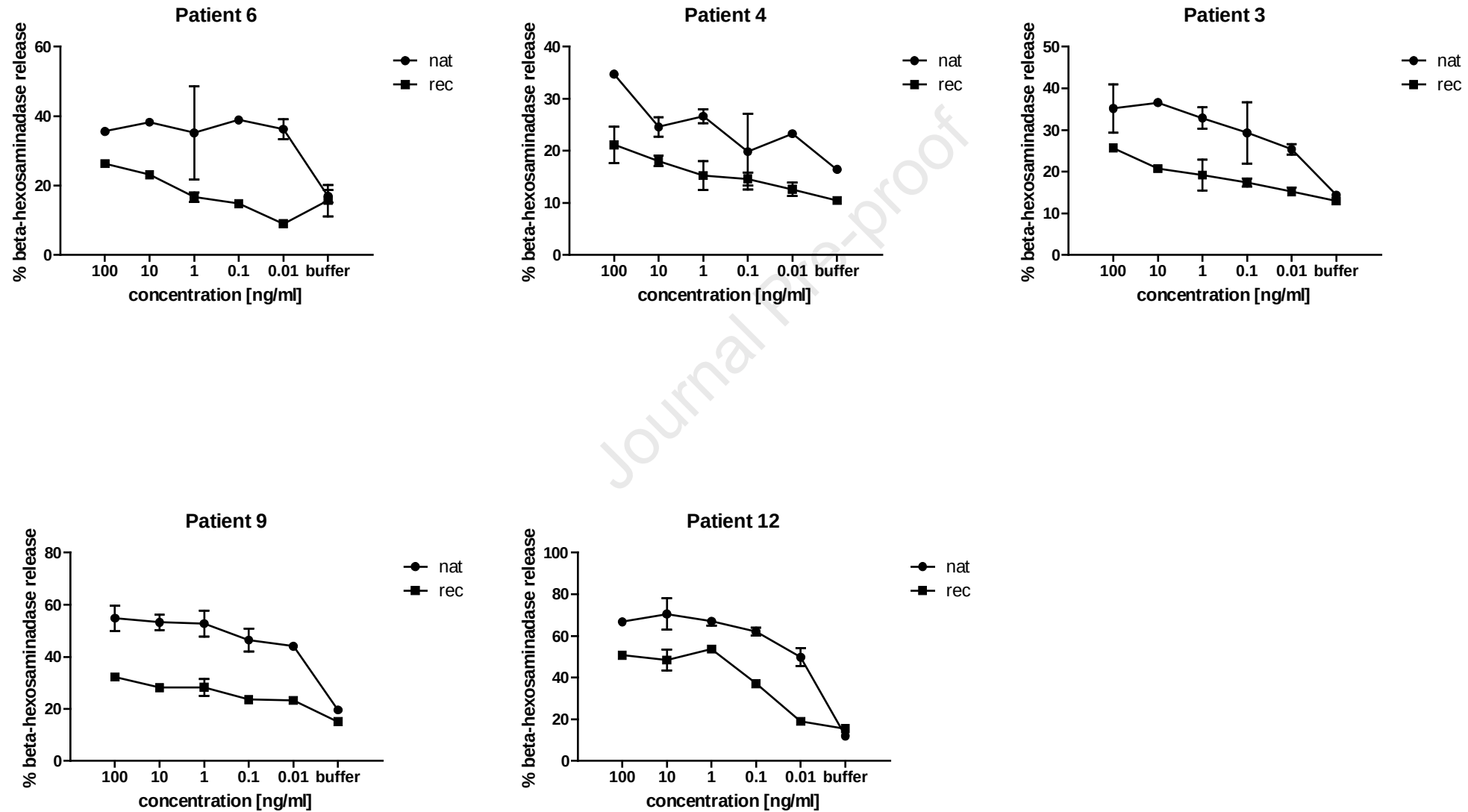


Fig E1.

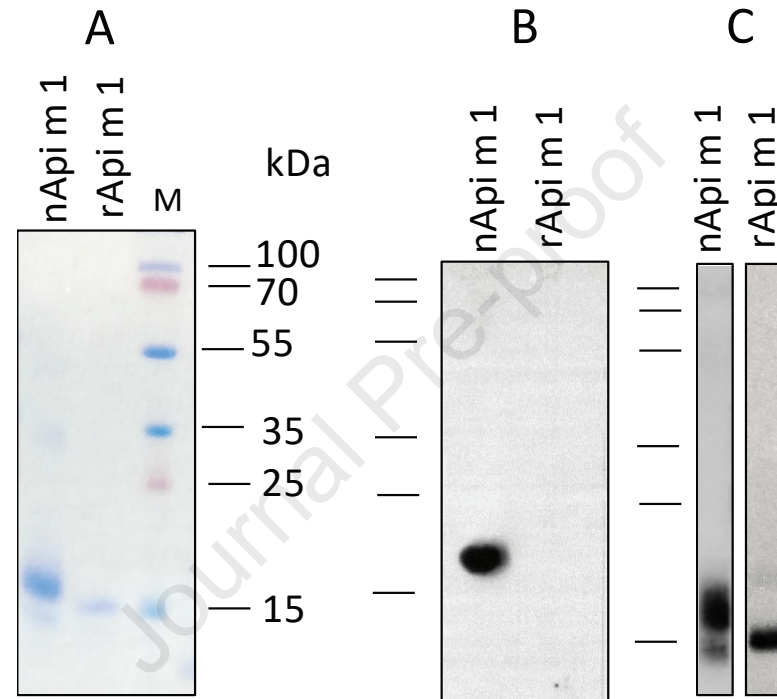


FIG E2.

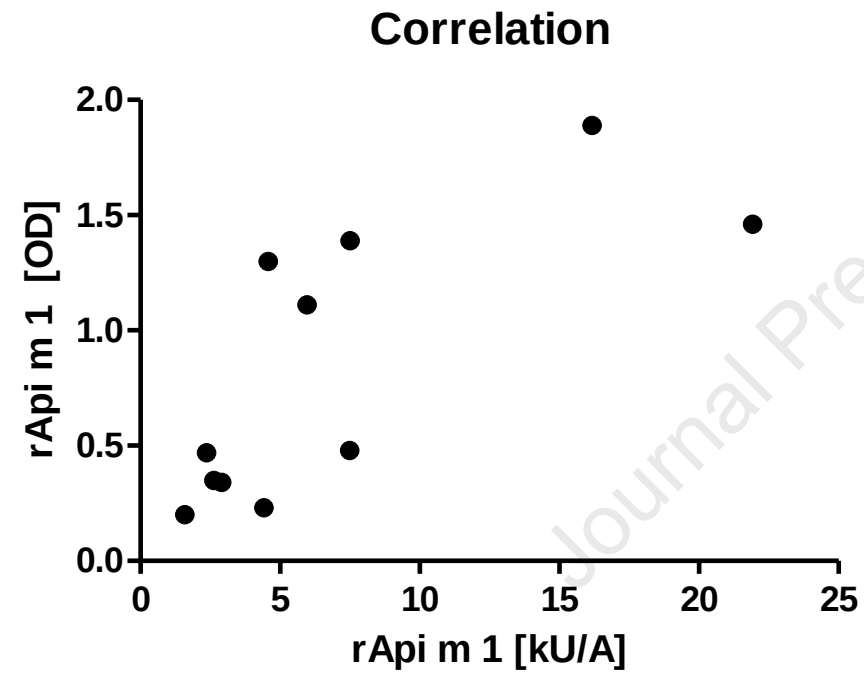


FIG E3.

