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Two galactose- α -1,3-galactose carrying peptidases from pork kidney mediate anaphylactogenic responses in delayed meat allergy

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Short title: Characterization of α -Gal carrying allergens in pork kidney

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Abstract

Background: Serum IgE-antibodies directed at galactose- α -1,3-galactose (α -Gal) are associated with a novel form of delayed anaphylaxis occurring upon consumption of red meat or innards. Pork kidney is known as the most potent trigger of this syndrome, but the culprit allergens have not yet been identified. The aim of this study was the identification and characterization of pork kidney proteins mediating delayed anaphylactic reactions through specific IgE to α -Gal.

Methods: A cohort of 59 patients with specific IgE to α -Gal was screened by immunoblot for IgE-reactive proteins in pork kidney. Proteins were identified by peptide mass fingerprinting. Isolated proteins were assayed in ELISA and ELISA inhibition, basophil activation and skin prick test.

Results: Several IgE-binding proteins of high molecular weight (100 - >200 kDa) were detected in pork kidney extracts by immunoblot using patient sera and an anti- α -Gal antibody. Two major IgE-binding proteins were identified as porcine angiotensin I converting enzyme (ACE I) and aminopeptidase N (AP-N). Reactivity of patient sera and anti- α -Gal antibody to both proteins was abolished by carbohydrate oxidation. The α -Gal IgE epitopes were resistant to heat denaturation. Pork kidney extract, isolated ACE I and AP-N were able to activate patient basophils and elicit positive responses in skin prick tests.

Conclusion: Two cell-membrane proteins carrying α -Gal epitopes were identified in pork kidney. For the first time, isolated meat proteins were shown to induce basophil activation in patients with delayed anaphylaxis to red meat providing further confirmation for the clinical relevance of these α -Gal-carrying proteins.

Key words:

Allergen, carbohydrate, galactose- α -1,3-galactose, red meat allergy, pork kidney

Abbreviations:

ACE I, angiotensin I converting enzyme; AP-N, aminopeptidase N; α -Gal, galactose- α -1,3-galactose

Introduction

Delayed anaphylaxis to red meat has been associated with specific IgE-antibodies to the carbohydrate epitope galactose- α -1,3-galactose (α -Gal).(1) Increasing numbers of cases are reported worldwide and there is strong evidence that tick bites constitute the major sensitization source. (2) The presence of specific IgE-antibodies to α -Gal constitutes an important health issue as patients are not only at risk for delayed anaphylaxis to red meat and gelatin-containing products, (3-5) but even more importantly to immediate type anaphylaxis to therapeutic antibodies of rodent origin (6) or to bovine-derived gelatin colloids administered as plasma substitutes. (5, 7) The use of bioprosthetic material such as heart valves of bovine and porcine origin may put highly sensitized patients at risk for peri- or postoperative hypersensitivity reactions. (8) Among other epitopes, α -Gal is known to constitute a major obstacle to xenotransplantation and different approaches exist to inactivate or mask these epitopes in xenogeneic tissues. (9)

Patients may react to a variety of mammalian meat products. Delayed urticarial or anaphylactic reactions were reported in different parts of the world. They occurred 2-6 hours after ingestion of red meat and were associated with eating beef, pork, lamb, rabbit, horse and kangaroo.(1, 5, 10) In Europe, beef and pork innards, in particular kidney, liver and tripe, are consumed traditionally. Earlier reports had shown that in particular pork kidney is rich in α -Gal. (11) After ingestion of pork kidney, symptoms were often severe and occurred within a shorter delay compared to previously described reactions to mammalian skeletal muscle meat. Prick-to-prick tests using raw or cooked pork kidney showed 100% sensitivity compared to raw pork (42-63%) and raw beef (33-38%).(12) A

common feature in all these studies was that patients were mostly adults with a mean age of 55 years, that symptoms often but not always appeared before onset of anaphylaxis and that most of the patients had no previous allergies. European studies in addition focussed on the consumption of innards and the role of cofactors such as physical exercise, alcohol or drug intake which were reducing the delay of reaction in patients with delayed red meat allergy. (11, 12).

Two recent publications identified several IgE- and α -Gal-reactive proteins from beef meat by two-dimensional gel electrophoresis and peptide mass fingerprinting techniques. (13, 14) However, in both studies the proteins were not isolated to further confirm their identity, to test their capacity of IgE binding or to show their clinical relevance using basophil activation or skin testing. The objective of the present study was to identify and isolate the proteins carrying IgE-reactive α -Gal-epitopes in pork kidney, a tissue which is particularly rich in α -Gal and which is known to be the most potent inducer of delayed food-induced anaphylaxis after consumption of meat products. Two cell-membrane-bound proteins with multiple glycosylation sites, both belonging to the metallopeptidase family, were recognized as IgE-reactive proteins carrying α -Gal-epitopes. For the first time, purified meat proteins were characterized in immunoassays using patient sera and α -Gal-specific reagents. Their potential relevance and allergenicity was further confirmed in IgE inhibition experiments, basophil activation assays and skin prick testing.

Material and methods

Patients and sera

Patients (n=59) were recruited on the basis of having either a clinically confirmed syndrome of delayed anaphylaxis to red meat with specific IgE to α -Gal or a clinically asymptomatic sensitization to α -Gal with specific IgE to α -Gal >1.5 kU_A/L (ImmunoCAP, Thermo Fisher Scientific, Uppsala, Sweden) (Table S1, in this manuscript's Online Repository).

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Patients were recruited from the outpatient clinic of the National Immunology-Allergology Unit at the Centre Hospitalier de Luxembourg (CHL) and the Allergology Unit at the Department of Dermatology in Tuebingen. The sera and data from patients with clinically asymptomatic sensitization to α -Gal were provided from an ongoing epidemiological study conducted at the Department of Dermatology in Tuebingen, assessing the prevalence of α -Gal sensitizations among forestry worker and hunters in Southern Germany. Three healthy individuals and one patient with pork-cat syndrome were included as controls.

Pork and beef meat and kidney were tested by prick-to-prick test. Purified α -Gal containing allergens (AP-N and ACE I) were diluted to 0.1, 0.5, 5 and 20 μ g/mL in human serum albumin diluent for skin prick testing (ALK-Abello, Wedel, Germany). Cetuximab (Erbix[®], Merck Serono, Darmstadt, Germany) was used at concentrations of 5-500 μ g/mL as described. (11) Histamine chloride (0.1%) was used as positive control and diluent as negative control. Reactions with a wheal having a diameter of ≥ 3 mm were rated positive.

The study was approved by the National Committee for Medical Research Ethics in Luxembourg and by the Ethics Commission of the University Medical Faculty in Tuebingen.

Reagents

Gal- α -1,3-Gal- β -1,4-GlcNAc-HSA (α -Gal-HSA; NGP2334) and Gal- α -1,3-Gal- β -1,4-GlcNAc (α -Gal, GN334), a linear trisaccharide, were obtained from Dextra Laboratories (Reading, UK). Bovine thyroglobulin was purchased from Sigma-Aldrich (Diegem, Belgium). Porcine ACE I and AP-N purified from pork kidney were obtained from Sigma and Merck-Millipore (Darmstadt, Germany), respectively. Mouse monoclonal anti-ACE I and rabbit polyclonal anti-AP-N antibodies raised against human ACE I and AP-N were from Santa Cruz Biotech (Heidelberg, Germany). The mouse monoclonal anti- α -Gal IgM antibody, clone M86, was obtained from Enzo Life Sciences (Lausen, Switzerland).

Protein extractions

Pork kidney was purchased at a local retailer and cut into small pieces. Lysis buffer (50 mM Tris HCl pH8) containing protease inhibitors (Protease Inhibitor Cocktail Tablets; Roche, Mannheim, Germany) was added and tissue was disrupted with beads (Tissue Lyser II, Qiagen; Hilden, Germany). Particles were eliminated by a centrifugation step for 10 min at 20 000 g.

Heated pork kidney was prepared by heating 500 mg of meat for 10 min at 95°C in a water bath. The extraction was performed with beads as described above. After the centrifugation step, the supernatant was collected for analysis by SDS-PAGE. Heat precipitated proteins were further extracted by a sonification step and by incubation in renaturing buffer (Roti-Restore, Roth, Karlsruhe, Germany) for 2 hours. Solubilized proteins were recovered for SDS-PAGE and immunoblot analysis after centrifugation at 20,000 g for 15 min. Pork kidney cell-membrane extracts were prepared using a subcellular protein fractionation kit (Pierce, Erembodegem, Belgium).

SDS-PAGE and immunoblotting

Proteins were separated under reducing conditions using 7.5 % SDS-PAGE and immunoblotted as described.⁽¹⁵⁾ For IgE-immunoblotting, patient sera were diluted 1:3 in blocking buffer (Tris-buffered saline, 0.05% Tween-20; 3% BSA (Sigma-Aldrich). Bound IgE was detected by anti-human IgE antibodies conjugated to alkaline phosphatase (Southern Biotech, Birmingham, AL, USA). Proteins carrying the carbohydrate α -Gal were visualized using a mouse monoclonal anti- α -Gal followed by incubation with a rat monoclonal anti-mouse IgM antibody labelled with alkaline phosphatase (Southern Biotech) and color development with nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl-phosphate (NBT/BCIP) (Promega, Leiden, The Netherlands).

Periodate treatment

IgE binding to carbohydrate epitopes was monitored by periodate treatment. Sodium periodate modifies carbohydrate structures by oxidation. Immunoblotted membrane strips with bound purified proteins or extracts were incubated for 2x 2 min in 50 mM sodium acetate (pH 4.5), followed by an 1 h incubation in the dark with 20 mM sodium periodate (NaIO₄), 50 mM sodium acetate. Strips were briefly washed in Tris buffered saline (TBS) containing 0.05% Tween-20, and then incubated in 1% glycine for 1 h. After a second washing step, strips were blocked with blocking buffer and incubated with antibodies and patient sera as stated above.

Lectin affinity chromatography

For purification of α -Gal carrying proteins, agarose bound *Griffonia simplicifolia* lectin I (Vector Laboratories, Peterborough, UK) was filled into a small polypropylen column (Qiagen), washed with 10 column volumes of HBS buffer (150 mM NaCl, 10 mM HEPES, 0.1 mM CaCl₂, pH 7.5). 1.5 mL of crude pork kidney extract were applied to the column. The column was washed with 2.5 volumes of HBS buffer and glycosylated proteins were eluted with glycoprotein elution buffer (Vector Laboratories).

Mass spectrometry and N-terminal sequencing

Protein bands were manually excised from Coomassie-stained SDS-PAGE and digested in gel with trypsin (sequencing grade, Roche). Digested peptides were analysed as described on a MALDI TOF/TOF instrument (Ultraflex I, Bruker Daltonics, Bremen, Germany) (15). Purified AP-N was subjected to N-terminal sequencing on a PPSQ protein sequencer (Shimadzu, Kyoto, Japan). (15)

Detection of specific IgE by ELISA and ELISA-inhibition

Specific IgE to α -Gal epitopes was quantified by ELISA, as previously described.(15) ACE I and α -Gal-HSA were coated at 2 μ g/mL, AP-N at 5 μ g/mL to microtiter plates (Nunc maxisorp, Roskilde, Denmark). Patient sera were diluted 1:5 in blocking buffer. A serum pool of 9 patients with low total IgE (range of total IgE 2.0-9.42 kU/L) was used as negative control.

For IgE-inhibition assays, sera were diluted 1:5 and incubated for 24h at 4°C with the respective inhibitors (Gal- α -1,3-Gal- β -1,4-GlcNAc 100 μ g/mL; pork kidney extract 200 μ g/mL) before being added to the microtiter plate.

Basophil activation assay

Basophil activation was performed using whole blood from 5 randomly selected patients within 24 h after blood collection using the FlowCAST kit (Bühlmann, Basel, Switzerland) essentially as described.(16) Relative cell counts of CD63 positive basophils were determined by flow cytometry (FACSCanto flow cytometer, BD, San José, California) upon stimulation with increasing amounts of protein (0.1 ng/mL - 100 μ g/mL).

Results

Detection of IgE-reactive proteins in pork kidney cell-membrane extracts

Protein extracts were prepared from pork kidney and immunoblotted with patient sera. In order to enhance signal strength, different cellular fractions were extracted and analyzed for α -Gal carrying proteins using an anti- α -Gal murine monoclonal antibody. Membrane fractions were found to be enriched in α -Gal epitopes (data not shown) and were used in subsequent immunoblotting experiments. Sera of a group of 59 patients with specific IgE antibodies to α -Gal, selected by a positive ImmunoCAP to the α -Gal marker bovine thyroglobulin, were analyzed by immunoblot for reactivity to pork kidney membrane extract (Fig. 1). At least 6 protein bands were recognized with

variable signal intensities by a majority of patients as well as by the anti- α -Gal monoclonal antibody. All IgE-reactive proteins were of high molecular weight: above 200 kDa, 180, 160, 120 and 100 kDa. The serum of a patient with cat-pork syndrome without specific IgE to α -Gal was used as a control. The serum did not react with pork kidney membrane extract, but as expected with serum albumin, a 66 kDa protein in whole kidney extract, which is absent in membrane extracts (Fig. 1).

IgE-reactivity is dependent on glycosylation

In order to discriminate IgE-reactivity to protein epitopes from reactivity directed to carbohydrates, protein extracts were treated on the membrane strip by sodium metaperiodate before incubation with patient sera (Fig. 2). Reactivity of the anti- α -Gal antibody and IgE-reactivity of patient sera to pork kidney membrane extract is completely abolished by the oxidation of carbohydrates.

Identification of α -Gal carrying proteins

Lectins are known to specifically bind carbohydrates. The *Griffonia (Bandeiraea) simplicifolia* lectin binds α -Galactose and N-acetylgalactosamine residues and it was used to enrich glycosylated allergens from pork kidney. Protein bands recognized by patient IgE and the anti- α -Gal antibody were analysed by mass spectrometry. Two transmembrane glycoproteins, aminopeptidase N (AP-N) and angiotensin I converting enzyme (ACE) I were identified (Table S2 in this manuscript's Online Repository). They correspond to the IgE-reactive bands detected at 160 and 180 kDa (Figure 1). Both proteins are known to be highly glycosylated and abundantly expressed in kidney epithelial cells. (17, 18) The carbohydrate content of porcine ACE I and AP-N was estimated to correspond to a molecular weight of 30 and 25 kDa, respectively. (17, 18)

ACE I and AP-N are major anti- α -Gal and IgE-reactive proteins present in pork kidney

Antibodies raised against ACE I and AP-N were used to detect the allergens in pork kidney membrane extract (Fig. 3A). Both antibodies strongly recognized protein bands at the same molecular weight as the anti- α -Gal antibody. ACE I and AP-N purified from pork kidney were

analyzed by Coomassie staining and immunoblot using patient serum P1 and anti- α -Gal antibody (Fig. 3B and 3C). As expected, a single band of about 180 kDa (17) was obtained by the protein stain and immunoblot for ACE I, using patient serum and anti- α -Gal antibody, whereas several bands were detected for AP-N. Intact AP-N migrates at 150 kDa, but upon purification, it readily degrades into 2 fragments previously determined as subunits B and C. (19) N-terminal (Edman) sequencing and MS/MS analysis confirmed that the band >120 kDa corresponds to uncleaved AP-N with an N-terminus starting at amino acid (aa) 62, the 95 kDa band is identical to subunit B, which has 11 potential glycosylation sites (data not shown). The 60 kDa protein is identified as co-purified porcine serum albumin. The N-terminus of the 50 kDa band starts at aa 574 and corresponds to the previously described subunit C. This C-terminal fragment is not recognized by patient IgE nor by the anti- α -Gal antibody. Thus its 3 putative glycosylation sites do probably not carry α -Gal-epitopes or they may not be accessible. Periodate treatment abolishes patient IgE and anti- α -Gal reactivity to all protein bands.

Reactivity to α -Gal is not affected by heating

Patients with delayed meat anaphylaxis have been reported to react positively in skin prick test both with raw and cooked meat.(12) Pork kidney was heated for 10 min at 95 °C before protein extraction. Although proteins with α -Gal reactivity precipitated during this step, they retained their carbohydrates and were not degraded (Fig. 3D).

Detection of ACE I and AP-N in pork and beef

ACE I and AP-N are conserved mammalian proteins. Amino acid sequence identities are between 84 and 88 % for porcine, bovine, horse and rabbit ACE I sequences and between 77-84 % for AP-N (data not shown). A number of potential glycosylation sites are present on all of these proteins. ACE I could be detected in pork kidney and at lower levels in pork meat. ACE I detection in bovine kidney was much weaker than in pork kidney and undetectable in bovine meat. (Figure S1 in this

manuscript's Online Repository). However the apparent lower ACE I levels in bovine tissue could also be attributed to the fact that the anti-ACE I antibody used was a monoclonal raised against human ACE I which might have a lower affinity to bovine ACE I. AP-N was strongly expressed in porcine and bovine kidney. Weak signals possibly corresponding to subunits B and C of AP-N were detected at a lower molecular weight in meat tissue of pork and beef.

IgE-antibodies directed to ACE I and AP-N are detectable by immunoassay and are inhibited by α -Gal

A number of patients with strong signals in IgE-immunoblot and specific IgE to α -Gal (n=40) were tested in ELISA for the presence of specific IgE to ACE I, AP-N and α -Gal-HSA (Fig. 4A and Fig S2 in this manuscript's Online Repository). Reactivity to purified pork kidney proteins in ELISA correlated well with reactivity to kidney membrane extract in IgE-immunoblot. Patient sera presented various IgE-reactivity profiles to both kidney proteins with AP-N being the most reactive. α -Gal-HSA was used as a control to assess reactivity to α -Gal. The batch used is specified to carry 21 carbohydrate residues per protein molecule. In order to further elucidate the nature of the IgE-reactivity, ELISA inhibition studies were performed with selected sera (Fig. 4B). We found evidence that the protein backbone of the carbohydrates influences IgE-recognition. This is illustrated by the different patient inhibition profiles. For patient sera P1 and P10, who are strongly reactive to α -Gal-HSA, inhibitions of IgE-reactivity to α -Gal-HSA are complete using α -Gal, but only partial when using pork kidney as inhibitor. In contrast, pork kidney is the best inhibitor for IgE-reactivity to ACE I and AP-N using sera P34 and P54, which were predominantly reacting to kidney proteins.

Allergenicity of AP-N and ACE I is confirmed by skin prick test and/or basophil activation

Five randomly selected patients were tested *in vivo* and *ex vivo* for reactivity to AP-N and ACE I. All 5 had a positive skin prick test to AP-N, but only 1 reacted to ACE I at the concentrations tested, which were in the normal range used for purified allergens (Table 1). (15, 20) Cetuximab was positive either in skin prick test at 50-500 μ g/mL or by intradermal testing.

The basophil activation assay was strongly positive for patients P51, P53 and P59 with AP-N, ACE I, and pork kidney extract (Fig. 5). Reactivity of patient P54 basophils was low, and only AP-N and pork kidney yielded clearly positive results. P52 was a non-responder. None of the proteins activated basophils of a control patient without specific IgE to α -Gal showing the IgE-dependency of the activation.

Discussion

In this study, we identified two cell-membrane-bound metallopeptidases as major α -Gal-carrying allergens in pork kidney. IgE-recognition was dependent on the presence of carbohydrates carrying the α -Gal epitope. Both proteins are heavily glycosylated cellular transmembrane proteins.(17, 18) AP-N, also known as CD13, is present on a number of cell types, but it is most abundantly expressed in enterocytes of the small intestine and on the brush border membrane of epithelial cells of kidney proximal tubules. (18) It plays a role in a variety of functions such as enzymatic cleavage of regulatory peptides (angiotensin III, kinins) and peptides issued from gastrointestinal digestion, endocytosis, viral receptor and signal regulation. (21) ACE I plays a crucial role in the angiotensin pathway.(22) Although ACE I is most abundant in kidney and lung, it is ubiquitously present in tissues and fluids. We have shown that ACE I and AP-N are abundant in pork kidney and that they are heat resistant. It has been speculated that the number of α -Gal epitopes in a tissue is related to the rapidity and severity of the clinical reaction.(11) Glycosylated AP-N and ACE I are also present in other consumed mammalian meats such as cattle, horse or rabbit. The presence of α -Gal epitopes in these tissues is likely and should be further investigated.

Several beef allergens have been described in two recent publications. Takahashi et al. reported 2 high molecular weight proteins of 240 and 140 kDa, laminin γ -1 and collagen α -1 (VI) chain to be IgE-reactive.(14) This reactivity was sensitive to deglycosylation. Apostolovic et al. analysed processed beef proteins and identified 7 beef allergens in the 26-47 kDa molecular weight range that were also recognized by the anti- α -Gal antibody and thus supposed to carry the α -Gal epitope.(13) Four of them were stable to heat treatment. However, none of those proteins has been isolated and further analysed to confirm their allergenicity. In our study on pork kidney allergens, we did not detect relevant IgE-reactive bands below 66 kDa (data not shown). It is therefore unlikely that some of the postulated beef allergens would also be allergens in pork kidney. So far, we have not analyzed the IgE-reactive bands above 200 kDa, we can only speculate if one of these could be identical to laminin.

In contrast to previous studies, we analyzed IgE-reactivity of purified meat proteins in ELISA and inhibition ELISA. IgE recognition profiles to AP-N, ACE I and α -Gal-HSA were patient dependent. There are several possible explanations to this observation. Although all patients recognized α -Gal, either on the ImmunoCAP as bovine thyroglobulin or purified kidney proteins or α -Gal-HSA, there were differences in reactivity which may either be explained by the contribution of the protein environment to the epitope, the diversity of sugars attached near the α -Gal-epitope or the number of α -Gal-epitopes. The presence of anti- α -Gal immunoglobulins of different subtypes (IgM, IgG, IgA) in patient serum may inhibit IgE-binding to some epitopes in individual patients. (23) The determination of the carbohydrate content of AP-N and ACE I will be the subject of further analyses. The different patient reactivity profiles may reflect α -Gal-epitopes originating from various sensitizing molecules which are present in our environment. It is commonly accepted that tick bites are the most likely primary sensitization source as documented by different reports from the US, Australia, Europe and Japan (24-27), but other, so far unknown sensitizers may play a role.

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Currently, neither skin prick tests nor the quantification of specific IgE directed against α -Gal nor the ratio of specific IgE/total IgE (table S1) do allow for differentiation of sensitized and clinically reactive patients. The in-depth analysis of α -Gal carrying IgE-epitopes and the role of the protein backbone are a prerequisite for the improvement of *in vitro* diagnosis of patients with delayed anaphylaxis to red meat.

Allergenicity of AP-N and ACE I was evaluated by basophil activation assay and skin prick test. Both allergens were able to stimulate basophil release at concentrations of 0.1-10 μ g/mL, approximately a factor 10-100 higher in comparison to assays with IgE directed against protein epitopes. (28, 29). AP-N elicited a positive skin response at ≤ 5 μ g/mL in four patients and at 20 μ g/mL in 1 patient whereas Cetuximab was only positive at much higher doses or by intradermal testing. Only 1 patient reacted to ACE I at the doses tested in skin prick test. It is possible that higher doses will be needed for a positive response as in the case of Cetuximab. The higher reactivity of AP-N in skin prick test and basophil activation test will be the subject of further investigations.

In conclusion, we have identified two important IgE-reactive α -Gal carrying allergens in pork kidney, a tissue which is known as a potent trigger of red meat-induced delayed anaphylaxis. IgE-reactivity is dependent on carbohydrates which are heat stable. Clinical relevance of AP-N and ACE I has been confirmed by basophil activation assay and skin prick testing.

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Author contributions:

All authors critically reviewed the manuscript. CH was involved in all stages of the project and wrote the manuscript; KS performed most experiments; JF, FH, CL, MM, BE and TB recruited patients and participated in data analysis and interpretation; CH, FH and MO designed the study.

Conflict of interest:

The authors declare no conflict of interest for the work presented in this study.

References

1. Commins SP, Satinover SM, Hosen J, Mozena J, Borish L, Lewis BD, et al. Delayed anaphylaxis, angioedema, or urticaria after consumption of red meat in patients with IgE antibodies specific for galactose-alpha-1,3-galactose. *J Allergy Clin Immunol* 2009;**123**(2):426-433.
2. Commins SP, Platts-Mills TA. Delayed anaphylaxis to red meat in patients with IgE specific for galactose alpha-1,3-galactose (alpha-gal). *Curr Allergy Asthma Rep* 2013;**13**(1):72-77.
3. Caponetto P, Fischer J, Biedermann T. Gelatin-containing sweets can elicit anaphylaxis in a patient with sensitization to galactose-alpha-1,3-galactose. *J Allergy Clin Immunol Pract* 2013;**1**(3):302-303.
4. Commins SP, Platts-Mills TA. Anaphylaxis syndromes related to a new mammalian cross-reactive carbohydrate determinant. *J Allergy Clin Immunol* 2009;**124**(4):652-657.
5. Mullins RJ, James H, Platts-Mills TA, Commins S. Relationship between red meat allergy and sensitization to gelatin and galactose-alpha-1,3-galactose. *J Allergy Clin Immunol* 2012;**129**(5):1334-1342.e1331.
6. Chung CH, Mirakhur B, Chan E, Le Q-T, Berlin J, Morse M, et al. Cetuximab-Induced Anaphylaxis and IgE Specific for Galactose-{alpha}-1,3-Galactose. *N Engl J Med* 2008;**358**(11):1109-1117.

7. Uyttebroek A, Sabato V, Bridts CH, De Clerck LS, Ebo DG. Anaphylaxis to succinylated gelatin in a patient with a meat allergy: galactose-alpha(1, 3)-galactose (alpha-gal) as antigenic determinant. *J Clin Anesth* 2014;**26**(7):574-576.
8. Mozzicato SM, Tripathi A, Posthumus JB, Platts-Mills TA, Commins SP. Porcine or bovine valve replacement in 3 patients with IgE antibodies to the mammalian oligosaccharide galactose-alpha-1,3-galactose. *J Allergy Clin Immunol Pract* 2014;**2**(5):637-638.
9. Naso F, Gandaglia A, Iop L, Spina M, Gerosa G. Alpha-Gal detectors in xenotransplantation research: a word of caution. *Xenotransplantation* 2012;**19**(4):215-220.
10. Jacquenet S, Moneret-Vautrin DA, Bihain BE. Mammalian meat-induced anaphylaxis: clinical relevance of anti-galactose-alpha-1,3-galactose IgE confirmed by means of skin tests to cetuximab. *J Allergy Clin Immunol* 2009;**124**(3):603-605.
11. Morisset M, Richard C, Astier C, Jacquenet S, Croizier A, Beaudouin E, et al. Anaphylaxis to pork kidney is related to IgE antibodies specific for galactose-alpha-1,3-galactose. *Allergy* 2012;**67**(5):699-704.
12. Fischer J, Hebsaker J, Caponetto P, Platts-Mills TA, Biedermann T. Galactose-alpha-1,3-galactose sensitization is a prerequisite for pork-kidney allergy and cofactor-related mammalian meat anaphylaxis. *J Allergy Clin Immunol* 2014;**134**(3):755-759.e751.
13. Apostolovic D, Tran TA, Hamsten C, Starkhammar M, Cirkovic Velickovic T, van Hage M. Immunoproteomics of processed beef proteins reveal novel galactose-alpha-1,3-galactose-containing allergens. *Allergy* 2014;**69**(10):1308-1315.
14. Takahashi H, Chinuki Y, Tanaka A, Morita E. Laminin gamma-1 and collagen alpha-1 (VI) chain are galactose-alpha-1,3-galactose-bound allergens in beef. *Allergy* 2014;**69**(2):199-207.
15. Hilger C, Dubey VP, Lentz D, Davril C, Revets D, Muller CP, et al. Male-specific submaxillary gland protein, a lipocalin allergen of the golden hamster, differs from the lipocalin allergens of Siberian and Roborovski dwarf hamsters. *Int Arch Allergy Immunol* 2015;**166**(1):30-40.

16. Eberlein B, Krischan L, Darsow U, Ollert M, Ring J. Double positivity to bee and wasp venom: improved diagnostic procedure by recombinant allergen-based IgE testing and basophil activation test including data about cross-reactive carbohydrate determinants. *J Allergy Clin Immunol* 2012;**130**(1):155-161.
17. Hooper NM, Turner AJ. Isolation of two differentially glycosylated forms of peptidyl-dipeptidase A (angiotensin converting enzyme) from pig brain: a re-evaluation of their role in neuropeptide metabolism. *Biochem J* 1987;**241**(3):625-633.
18. Sjostrom H, Noren O, Olsen J. Structure and function of aminopeptidase N. *Adv Exp Med Biol* 2000;**477**:25-34.
19. Wacker H, Lehky P, Vanderhaeghe F, Stein EA. On the subunit structure of particulate aminopeptidase from pig kidney. *Biochim Biophys Acta* 1976;**429**(2):546-554.
20. Sirvent S, Canto B, Cuesta-Herranz J, Gomez F, Blanca N, Canto G, et al. Act d 12 and Act d 13: two novel, masked, relevant allergens in kiwifruit seeds. *J Allergy Clin Immunol* 2014;**133**(6):1765-1767.e1764.
21. Mina-Osorio P. The moonlighting enzyme CD13: old and new functions to target. *Trends Mol Med* 2008;**14**(8):361-371.
22. Guang C, Phillips RD, Jiang B, Milani F. Three key proteases--angiotensin-I-converting enzyme (ACE), ACE2 and renin--within and beyond the renin-angiotensin system. *Arch Cardiovasc Dis* 2012;**105**(6-7):373-385.
23. Galili U. The alpha-gal epitope and the anti-Gal antibody in xenotransplantation and in cancer immunotherapy. *Immunol Cell Biol* 2005;**83**(6):674-686.
24. Van Nunen SA, O'Connor KS, Clarke LR, Boyle RX, Fernando SL. An association between tick bite reactions and red meat allergy in humans. *Med J Aust* 2009;**190**(9):510-1.
25. Commins SP, James HR, Kelly LA, Pochan SL, Workman LJ, Perzanowski MS et al. The relevance of tick bites to the production of IgE antibodies to the mammalian oligosaccharide galactose- α -1,3-galactose. *J Allergy Clin Immunol* 2011;**127**(5):1286-93.e6.

26. Hamsten C, Starkhammar M, Tran TA, Johansson M, Bengtsson U, Ahlén G et al. Identification of galactose- α -1,3-galactose in the gastrointestinal tract of the tick *Ixodes ricinus*; possible relationship with red meat allergy. *Allergy* 2013;**68**(4):549-52.
27. Chinuki Y, Ishiwata K, Yamaji K, Takahashi H, Morita E. *Haemaphysalis longicornis* tick bites are a possible cause of red meat allergy in Japan. *Allergy* 2015 Nov 9. doi: 10.1111/all.12804. [Epub ahead of print].
28. Hilger C, Kler S, Arumugam K, Revets D, Muller CP, Charpentier C, et al. Identification and isolation of a Fel d 1-like molecule as a major rabbit allergen. *J Allergy Clin Immunol* 2014;**133**(3):759-766.
29. Blank S, Seismann H, Michel Y, McIntyre M, Cifuentes L, Braren I, et al. Api m 10, a genuine *A. mellifera* venom allergen, is clinically relevant but underrepresented in therapeutic extracts. *Allergy* 2011;**66**(10):1322-1329.

Figure legends

Figure 1: Detection of IgE-reactive proteins in pork kidney protein extract

Patient sera with specific IgE to α -Gal were analysed for IgE reactivity to pork kidney membrane extract by immunoblot. The serum of an albumin-reactive cat-pork allergic patient (CP) was used as control. Numbered lanes correspond to patients in Table S1. α Gal, monoclonal mouse anti- α -Gal antibody; N, neg control for anti- α -Gal antibody; N1, N2 = negative control sera; M, molecular weight marker

Figure 2: IgE-reactivity to pork kidney is dependent on glycosylation

IgE-reactivity of patient sera to pork kidney membrane extract was analysed without (-) or after oxidation of carbohydrates by sodium periodate (NaIO_4) (+). Numbered lanes correspond to patients in Table S1. α Gal, anti- α -Gal antibody; M, molecular weight marker.

Figure 3: AP-N and ACE I are IgE- and α -Gal-reactive proteins present in pork kidney

Monospecific antibodies detect AP-N and ACE I in immunoblot of pork kidney membrane extract (A). Purified AP-N and ACE I are analysed by Coomassie-stained SDS-PAGE (B) and immunoblot (C) using patient serum P1 and anti- α -Gal antibody without (-) or after (+) oxidation of carbohydrates by sodium periodate (NaIO_4) treatment. Resistance to cooking is analysed by detection of α -Gal epitopes in raw and cooked pork kidney extracts in immunoblot using anti- α -Gal antibody (D). M, molecular weight marker (kDa); RK, raw pork kidney extract, CK_s, soluble proteins extracted from cooked pork kidney; CK_p, heat-precipitated proteins from cooked pork kidney. Arrows mark IgE and α -Gal reactive protein bands in the Coomassie-stained gel

Figure 4: IgE-quantification and IgE-inhibition experiments with AP-N, ACE I and α -Gal-HSA

Sera from 18 patients with specific IgE to α -Gal (1-59), 2 patients with cat-pork syndrome (CP 1 and 2) and a non-allergic patient pool (N) were analyzed for the presence of IgE-antibodies to AP-N, ACE I and α -Gal-HSA by immunoassay (A). Specific IgE of patients P1, P10, P34 and P54 directed to AP-N, ACE I and α -Gal-HSA were detected without (no inh) or with inhibition with 100 $\mu\text{g}/\text{mL}$ α -Gal (Gal- α -1,3-Gal- β -1,4-GlcNAc) or 200 $\mu\text{g}/\text{mL}$ pork kidney extract (PK) (B).

Figure 5: Basophil activation assay

Patients' basophils were sensitized with increasing concentrations of α -Gal containing proteins ACE I, AP-N, α -Gal-HSA, Cetuximab and pork kidney extract. Non-glycosylated human serum albumin (HSA) served as negative control, anti-Fc ϵ RI as positive stimulation control. Relative cell counts of CD63⁺ basophils were determined by flow cytometry. Negative control, patient without specific IgE to α -Gal.

Figure S1: ACE I and AP-N are present in beef and pork

Raw beef (BM) and pork (PM) meat extracts, beef (BK) and pork (PK) kidney extracts were analysed by Coomassie stain (A) and immunoblot (B) using anti-ACE I and anti-AP-N antibodies. M, molecular weight marker (kDa).

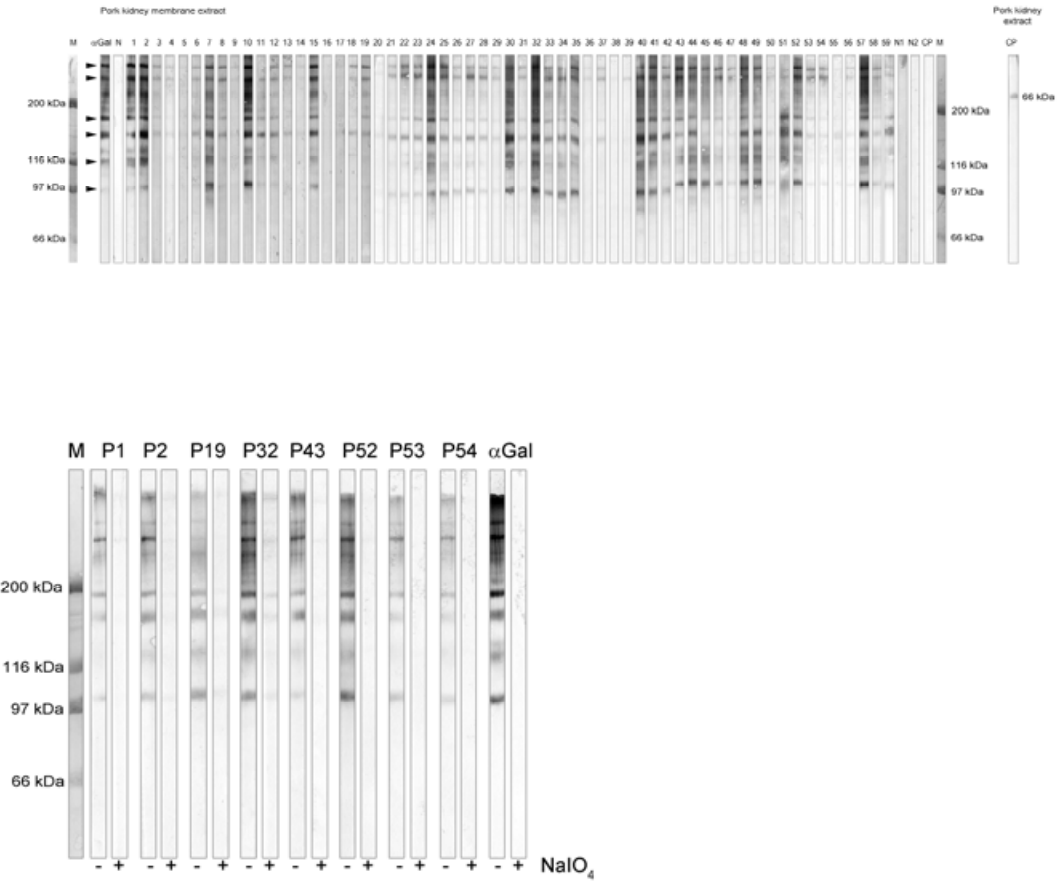
Figure S2: Heat map of patients' IgE reactivity to AP-N, ACE I, α -Gal-HSA and bovine thyroglobulin (α -Gal ImmunoCAP) by immunoassay. Patients 1-59 have specific IgE to α -Gal (1-59), patients CP 1 and CP 2 have cat-pork syndrome. N is a pool of non-allergic patient sera (N).

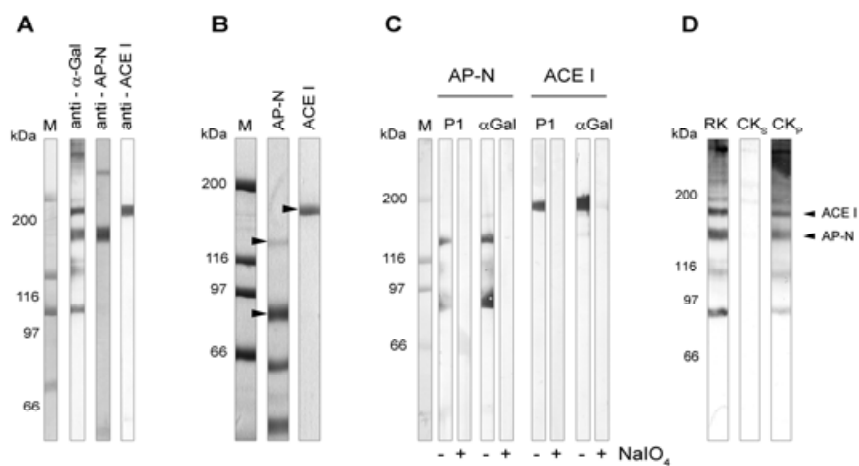
Table 1: Results of skin prick tests performed with different α -Gal carrying proteins and foods

	Patient 51	Patient 52	Patient 53	Patient 54	Patient 59
Wheal size	(mm)	(mm)	(mm)	(mm)	(mm)
AP-N 0.1 μ g/mL	nd	neg	neg	neg	5
5 μ g/mL	3	neg	5	4.5	nd
20 μ g/mL	3	4	nd	nd	nd
ACE I 5 μ g/mL	neg	neg	neg	neg	4
20 μ g/mL	neg	neg	neg	neg	nd
Pork kidney ¹	3	6.5	9	6	7
Beef kidney ¹	neg	neg	7.5	3	5
Pork ¹	nd	neg	neg	neg	5
Beef ¹	3	neg	3.5	5	4.5
Cetuximab 50 μ g/mL	neg	nd	4	neg	nd
Cetuximab 500 μ g/mL	3	3	nd	neg	5.5
Cetuximab ID 5 μ g/mL	nd	8	nd	5	nd

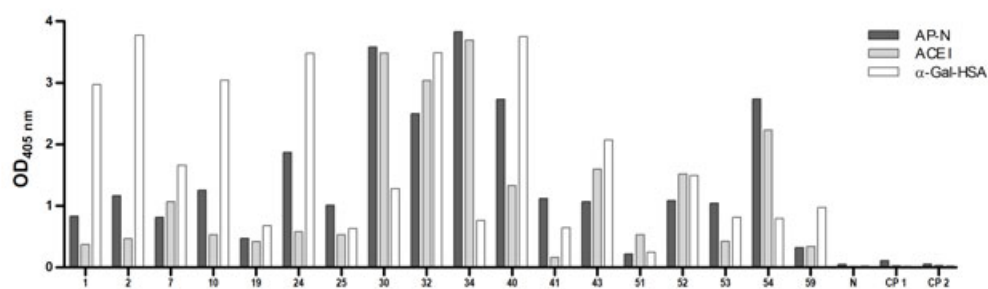
¹ prick-to-prick

ACE I, angiotensin I converting enzyme; AP-N, aminopeptidase N

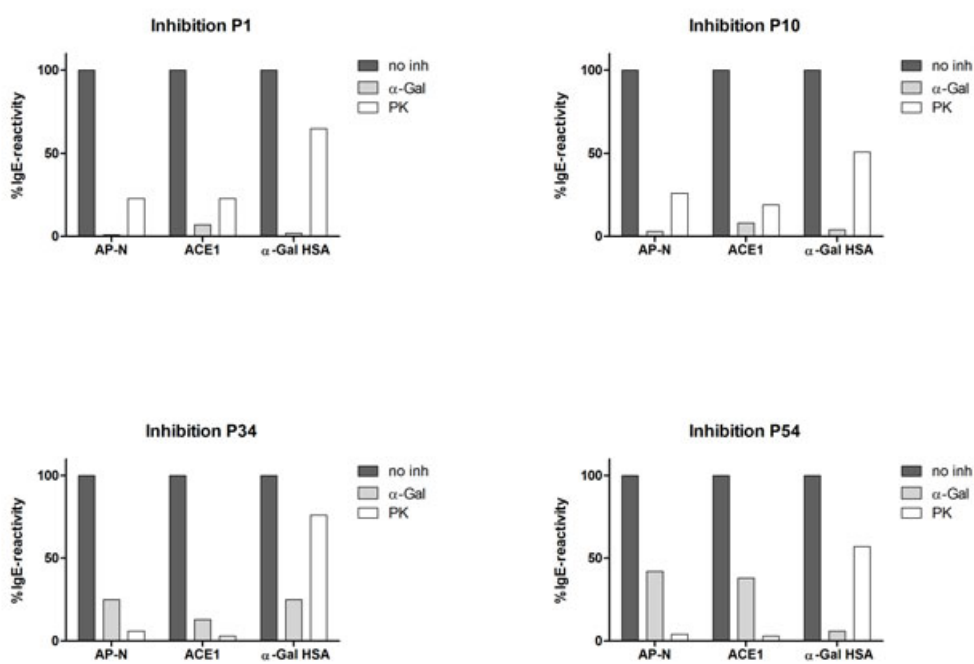


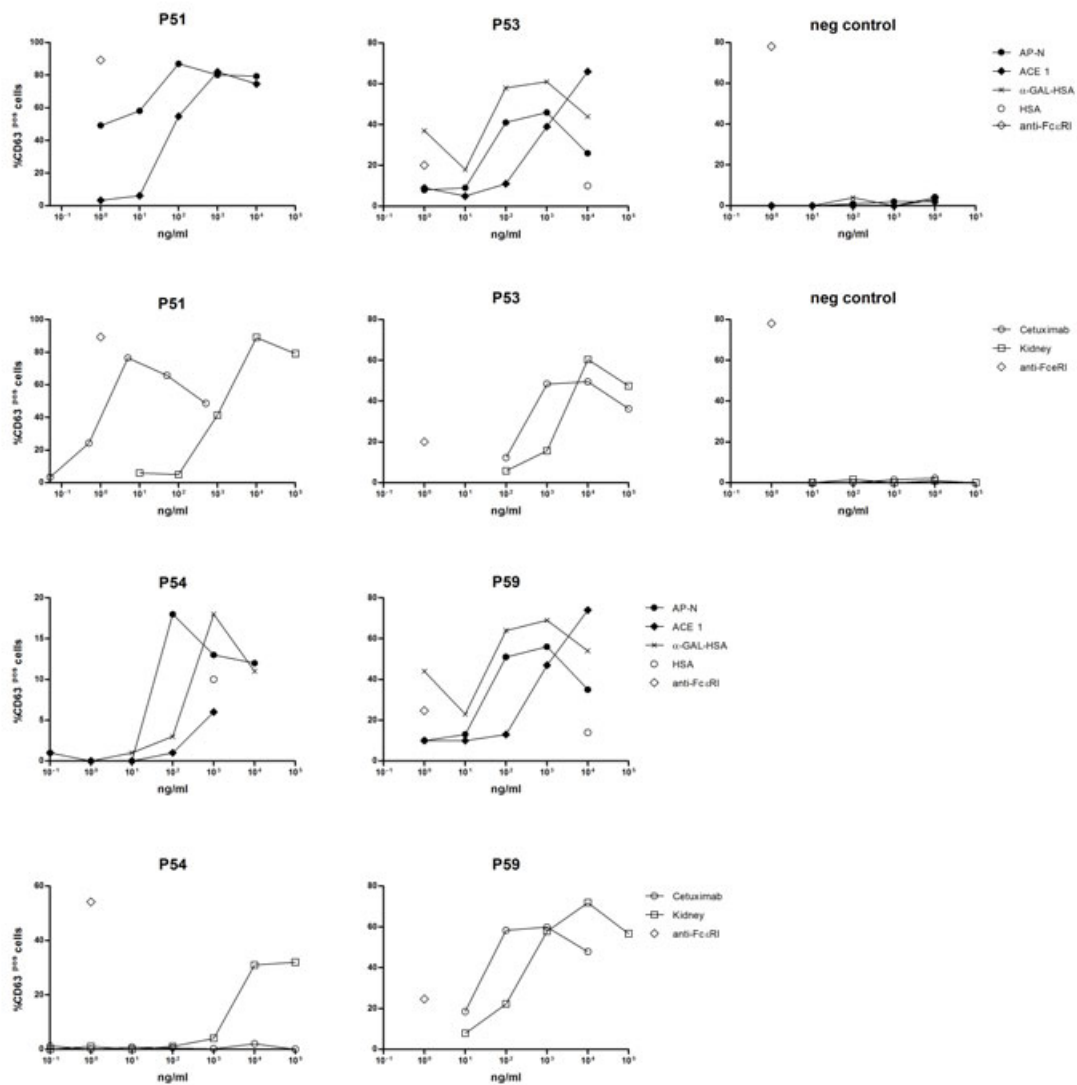


A

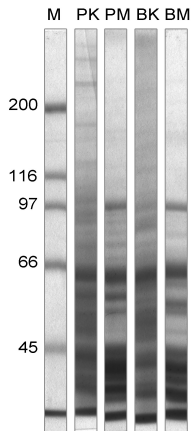


B

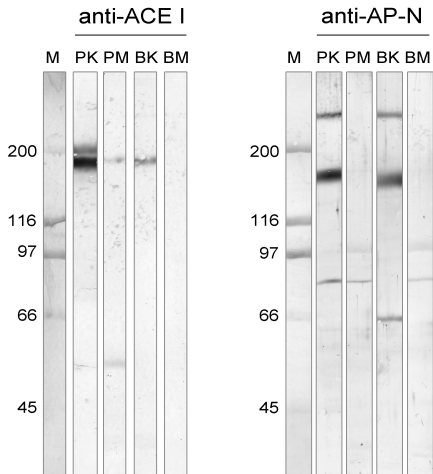




A



B



Patient	AP-N	ACE I	α -Gal-HSA	α -Gal ImmunoCAP o215
1	0.83	0.37	2.97	69.30
2	1.17	0.46	3.77	67.60
7	0.81	1.06	1.66	14.00
8	0.16	0.19	0.26	2.87
9	0.20	0.19	0.31	15.50
10	1.26	0.53	3.04	60.10
11	0.27	0.38	0.80	65.90
12	0.17	0.11	0.64	9.13
13	0.13	0.03	0.43	84.10
15	0.10	0.00	0.66	45.60
18	0.13	0.04	0.34	11.80
19	0.47	0.42	0.68	48.50
20	0.77	0.70	0.28	3.50
21	0.37	0.30	1.12	16.10
22	0.57	0.24	1.09	16.10
23	0.18	0.06	0.60	24.60
24	1.87	0.58	3.48	67.30
25	1.01	0.53	0.63	23.00
26	0.57	0.37	0.35	5.00
27	0.94	0.41	0.91	11.70
28	0.27	0.00	1.06	21.10
30	3.58	3.48	1.28	6.10
31	0.25	0.21	0.46	6.60
32	2.50	3.04	3.49	87.30
33	0.46	0.57	0.67	19.60
34	3.83	3.69	0.76	16.40
40	2.73	1.33	3.75	85.70
41	1.12	0.16	0.65	11.90
43	1.07	1.60	2.07	43.30
44	1.38	0.67	3.56	73.00
45	0.40	0.28	0.52	13.20
46	0.42	0.62	1.19	15.40
48	0.41	0.26	0.91	19.30
49	1.59	0.84	2.93	55.10
51	0.22	0.53	0.25	1.62
52	1.09	1.52	1.49	61.00
53	1.04	0.43	0.81	14.00
54	2.74	2.23	0.79	9.60
58	0.11	0.01	0.37	46.00
59	0.32	0.34	0.97	20.00
N	0.05	0.01	0.02	<0.1
CP 1	0.11	0.02	0.02	<0.1
CP 2	0.06	0.03	0.02	<0.1

IgE reactivity
(OD₄₀₅ nm)

	negative
	0.10-0.49
	0.50-0.99
	1.00-1.99
	2.00-3.99

ImmunoCAP
(kU_A/L)

	<0.1
	0.1-3.49
	3.5-17.0
	17.1-49.9
	50 - >100

Table S1: Clinical patient data

Patient no	Age	Sex	History of tick bite(s)	Meat eliciting symptoms	Delay of symptoms (hr)	Anaphylaxis according to Ring and Messmer (grade) [20,21]	Allergen sources tested in skin test*		α -GAL kU _A /L	Total IgE kU _A /L	Ratio anti-~Gal IgE/ tot IgE
							Positive skin test	Negative skin test			
1	47	M	yes	PK, P, B, V	< 1	2	PK, BK, P, G	B, Mi	69.3	594	0.117
2	61	M	yes	PK, B	n.d.	1	PK, BK, P, G	B, Mi	67.6	419	0.161
3	44	M	yes	no symptoms	n.d.	n.a.	n.a.		5.6	719	0.008
4	55	M	yes	P	> 3	2	PK, P, B, G	BK, Mi	2.8	255	0.011
5	48	M	yes	no symptoms	n.a.	n.a.	PK, G	BK, P, B	1.9	74.1	0.026
6	71	M	yes	no symptoms	n.a.	n.a.	PK, B	BK, P, G	1.4	86	0.016
7	83	M	yes	no symptoms	n.a.	n.a.	PK, BK, P, B ;	G	14.0	134	0.104
8	70	F	n.d.	PK	> 3	3	n.d.		2.9	42.2	0.069
9	32	M	yes	PK	1-3	2	PK, BK, G	P, B, Mi	15.5	167	0.093
10	66	M	no	PK, P	< 1	1	PK, BK, G	P, B, Mi	60.1	761	0.079
11	57	F	yes	PK, P	n.d.	1	PK, BK, G	P, B, Mi	65.9	148	0.445
12	55	M	yes	PK, P, B	> 3	3	n.d.		9.13	116	0.079
13	40	F	yes	PK, P	< 1	3	PK, G	BK, P, B, Mi	84.1	955	0.088
14	53	M	yes	PK	n.d.	1	PK, BK, G	P, B, Mi	1.6	74.9	0.021
15	57	M	yes	PK, P, B	< 1	2	PK, BK, P, B, G	Mi	45.6	401	0.114
16	65	F	yes	PK, M	1-3	3	PK, BK, G	P, B, Mi	9.2	74.5	0.123
17	63	F	yes	PK, P, B, M	< 1	2	PK, BK,	P, B, Mi ; G	2.1	1099	0.002
18	73	M	yes	PK, P	1-3	2	PK, BK, G	P, B, Mi	11.8	46.6	0.253
19	76	M	n.d.	PK	> 3	2	PK, BK, P, B,	Mi ; G	48.5	n.a.	
20	43	F	yes	PK	n.d.	1	n.d.		3.5	59.2	0.059
21	58	M	yes	no symptoms	n.a.	n.a.	PK, BK, P, B, G		16.1	192	0.084
22	51	M	yes	no symptoms	n.a.	n.a.	n.a.		16.1	58.1	0.277
23	49	M	yes	no symptoms	n.a.	n.a.	PK, P, B, G	BK	24.6	303	0.081
24	48	M	yes	no symptoms	n.a.	n.a.	n.a.		67.3	647	0.104
25	40	M	yes	no symptoms	n.a.	n.a.	n.a.		23.0	124	0.185
26	44	M	yes	no symptoms	n.a.	n.a.	n.a.		5.0	40.2	0.124
27	56	M	yes	no symptoms	n.a.	n.a.	BK, P, G	PK, B	11.7	117	0.100
28	52	M	yes	no symptoms	n.a.	n.a.	PK, BK, G	P, B	21.1	814	0.026
29	18	M	yes	no symptoms	n.a.	n.a.	n.a.		9.3	532	0.017
30	22	M	yes	no symptoms	n.a.	n.a.	n.a.		6.1	51.8	0.118

31	49	M	yes	no symptoms	n.a.	n.a.	n.a.		6.6	125	0.053
32	52	M	yes	no symptoms	n.a.	n.a.	n.a.		87.3	793	0.110
33	50	M	yes	no symptoms	n.a.	n.a.	n.a.		19.6	220	0.089
34	49	M	yes	no symptoms	n.a.	n.a.	n.a.		16.4	297	0.055
35	70	M	yes	P, B	> 3	3	PK, BK, G	Mi	3.9	149	0.026
36	60	M	n.d.	no symptoms	n.a.	n.a.	n.a.		1.5	280	0.005
37	60	M	yes	no symptoms	n.a.	n.a.	n.a.		5.7	247	0.023
38	57	M	yes	P	> 3	1	PK, BK, G	P, B, Mi	0.7	730	0.001
39	56	M	yes	no symptoms	n.a.	n.a.	n.a.		17.5	730	0.024
40	69	F	yes	B	> 3	2	PK, BK, P, B, G	Mi	85.7	252	0.340
41	73	F	yes	PK, P, B	> 3	2	PK, BK, B, G	P, Mi	11.9	163	0.073
42	59	M	yes	P, B, M, V	> 3	1	PK, BK, B, G	P, Mi	16.3	104	0.157
43	44	F	yes	P, B	> 3	2	PK, BK, P, B, G	Mi	43.3	132	0.328
44	38	M	n.d.	P, B	> 3	2	n.d.		73.0	134	0.545
45	55	M	yes	PK	> 3	2	PK, BK, P, B, G	Mi	13.2	29.9	0.441
46	50	F	n.d.	PK	1-3	1	n.d.		15.4	257	0.060
47	52	M	yes	PK	< 1	2	PK, G	BK, P, B, Mi	9.3	106	0.088
48	57	M	yes	B, M	> 3	2	PK, BK, P, B	Mi, G	19.3	114	0.169
49	74	M	yes	PK, B	< 1	3	PK, BK, P, G	B, Mi	55.1	200	0.276
50	77	M	yes	no symptoms	n.a.	n.a.	PK, BK, B ; G	P,	4.0	1086	0.004
51	47	M	no	PK, B	10 min, 1-3h	3	PK, B, G, V	P, BK, Mi	1.6	38.1	0.042
52	70	M	no	P, B	20-60 min	1	PK, BK, B	P, Mi	46.0	800	0.058
53	26	F	yes	P, B	1-6 h	1	PK, BK, B	P	9.0	177	0.051
54	16	M	yes	Mi, G	20-30 min	2	PK, B, Mi	P, BK	2.7	312	0.009
55	21	M	yes	B	6-8 h	1	PK, B	P	3.8	n.d.	
56	51	M	n.d.	P, B	3-4 h	1	n.d.	n.d.	3.9	305	0.013
57	43	M	n.d.	P, B, M	3-4 h	3	n.d.	n.d.	138.0	562	0.246
58	46	M	n.d.	P, B	4 h	1	n.d.	n.d.	46.0	135	0.341
59	43	M	yes	B, Mi	3-4 h	2	PK, BK, P, B, M, Mi, G		20.0	151	0.132

M, male; F, female; n.a., not applicable; n.d., no data

B, beef; BK, beef kidney; G, gelatin; M, mutton; Mi, cow's milk; P, pork; PK, pork kidney; V, venison

* A skin prick test was rated positive if wheal size was ≥ 3 mm, for ID ≥ 6 mm. Gelatin was tested by intradermal skin test. Grey shading: patient with clinical symptoms upon ingestion of red meat

Table S2: Identification of α -Gal containing proteins by peptide mass fingerprint after in-gel tryptic digestion

	NCBI database accession No	MW (kDa), pI	Score	Sequence coverage	Number of unique peptides	Predicted N- linked glycosylation sites
Aminopeptidase N (AP-N)	NP_999442	108.70; 5.15	172	39.9 %	27	14
Angiotensin- converting enzyme I (ACE I)	NP_001077410	150.54; 6.23	138	27.3 %	31	15

NCBI, National Center for Biotechnology Information