

PROF. MARGITTA WORM (Orcid ID : 0000-0002-3449-1245)

DR. ARNE HOMANN (Orcid ID : 0000-0002-2977-6174)

Article type : Original Article: Experimental Allergy and Immunology

Editor : Reto Crameri

Meat allergy associated with α -Gal – Closing diagnostic gaps by anti- α -Gal IgE immune profiling

U. Jappe^{1,2}, S. Minge¹, B. Kreft³, A. Ludwig⁴, B. Przybilla⁵, A. Walker⁵, R. Varga⁵, P. Seidel⁶, T. Biedermann^{6,7}, W. Anemüller⁸, A. Kromminga⁹, F. Ruëff⁵, H. Merk¹⁰, N. Wagner¹¹, R. Treudler¹², M. Worm¹³, I. Waldmann¹⁴, J. Saloga¹⁵, W. M. Becker¹, T. Goldmann¹⁶, T. A. Platts-Mills¹⁷, A. Homann¹

¹Division of Clinical and Molecular Allergology, Priority Research Area Asthma & Allergy, Research Center Borstel, Airway Research Center North (ARCN), Member of the German Center for Lung Research (DZL), Borstel, Germany, ²Interdisciplinary Allergy Division, Dept. of Internal Medicine, University of Lübeck, Germany, ³Dept. of Dermatology and Venereology, Martin-Luther-University Halle-Wittenberg, Halle (Saale), Germany, ⁴Dept. of Dermatology and Allergology, Zentralklinikum Augsburg, Germany, ⁵Dept. of Dermatology, Ludwig-Maximilians University, Munich, Germany, ⁶Dept. of Dermatology, University of Tübingen, Tübingen, Germany, ⁷Dept. of Dermatology and Allergology, Technical University of Munich, Germany, ⁸Dept. of Dermatology, University of Lübeck, Lübeck, Germany, ⁹IPM Biotech, Hamburg, Germany, ¹⁰Dept. of Dermatology, RWTH Aachen University, Aachen, Germany, ¹¹Dept. of Dermatology, University of Darmstadt, Darmstadt, Germany, ¹²Dept. of Dermatology, Venereology and Allergology, University of Leipzig, Leipzig, Germany, ¹³Dept. of Dermatology and Allergy, Allergy Center Charité, Berlin, Germany, ¹⁴Dermatology & Allergology, St. Bernward Klinik, Hildesheim, Germany, ¹⁵Dept. of

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/all.13238

This article is protected by copyright. All rights reserved.

Dermatology, University of Mainz, Mainz, Germany, ¹⁶Pathology of the University Medical Center Schleswig-Holstein (UKSH), Campus Lübeck and Research Center Borstel, Airway Research Center North (ARCN), Member of the German Center for Lung Research (DZL), Borstel, Germany, ¹⁷University of Virginia Asthma and Allergic Diseases Center, Charlottesville, USA

Corresponding author:

Prof. Dr. Uta Jappe

Division of Clinical and Molecular Allergology

Research Center Borstel

Parkallee 35

23845 Borstel

Germany

Tel.: +49 4537 1887406

Email: ujappe@fz-borstel.de

Short title: anti- α -Gal IgE immune profiling

Keywords: alpha-Gal, bovine thyroglobulin, cetuximab, delayed anaphylaxis, meat allergy

Abbreviations:

α -Gal – galactosyl- α -(1,3)-galactose, for details see also Fig. 1

Bos d TG – *Bos domesticus* (bovine) thyroglobulin

BSA – bovine serum albumin

CTX - cetuximab

Hom s - *Homo sapiens*

HRP – horseradish peroxidase

HSA – human serum albumin

IFX - infliximab

Mus m - *Mus musculus*

Neu5Ac – N-acetyl neuraminic acid

Neu5Gc - N-glycolyl neuraminic acid

TBS – Tris-buffered saline

MA –meat allergy

IA – idiopathic anaphylaxis

HC – healthy control

Abstract

Background: Glycoproteins and glycolipids of some mammalian species contain the disaccharide galactosyl- α -(1,3)-galactose (α -Gal). It is known that α -Gal is immunogenic in humans and causes glycan-specific IgG and also IgE responses with clinical relevance. α -Gal is part of the IgE-reactive monoclonal therapeutic antibody cetuximab and is associated with delayed anaphylaxis to red meat. In this study, different alpha-Gal-containing analytes are examined in singleplex and multiplex assays to resolve individual sensitization patterns with IgE against α -Gal.

Methods: Three serum groups, α -Gal-associated meat allergy (MA) patients, idiopathic anaphylaxis (IA) patients with suspected meat allergy and non-meat-allergic healthy control individuals (HC), were analyzed via singleplex allergy diagnostics and a newly established immunoblot diagnostic system. The new dot blot detection system resolved individual IgE sensitization profiles for α -Gal-containing analytes cetuximab, bovine thyroglobulin and HSA-conjugated α -Gal.

Results: Singleplex allergy diagnostics using the α -Gal-analytes cetuximab and bovine thyroglobulin confirms the history of meat allergy patients in 91% and 88% of the cases, respectively. A novel dot-blot-based assay system for the detection of IgE against α -Gal reveals individual IgE sensitization profiles for α -Gal-containing analytes. An α -Gal-associated IgE cross-reactivity profile (IgE against cetuximab, bovine thyroglobulin and HSA- α -Gal) was identified, which is associated with meat allergy.

Conclusions: Detection of individual sensitization patterns with different α -Gal-containing analytes provides the basis for an individual allergy diagnosis for α -Gal sensitized patients. Higher amounts of α -Gal in pork and beef innards compared to muscle meat as indicated by a higher staining intensity are a plausible explanation for the difference in allergic symptom severity.

Introduction

Clinical reactions against the therapeutic monoclonal antibody cetuximab (CTX) used for cancer treatment are severe, sometimes even fatal and are associated with IgE against galactosyl- α -(1,3)-galactose (α -Gal) (1). Meat allergy with particular clinical features was first observed in the USA and Australia (2,3) and has also been investigated in Sweden, Italy, Austria and Germany (4–7). It has been found that anti- α -Gal IgE plays a prominent role in meat allergy and severe allergy symptoms that sometimes develop with a delay of several hours (1,2,8,9). The intravenous application of CTX is especially dangerous for individuals that are already sensitized to α -Gal, potentially via tick bites (10,11). Before the association between meat allergy and IgE to α -Gal was discovered, meat allergy was diagnosed rarely, and only few studies and case reports were published describing the non-glycosylated bovine serum albumin (BSA, Bos d 6) as the most immunogenic protein (8). However, clinical allergologists had the suspicion from patients' history that in some cases meat allergy could not be properly verified by conventional diagnostics like skin prick tests with commercial reagents although the patient's allergy history was convincing (12). Thus, there is a need for diagnostic systems that are reliably detecting IgE against cross-reactive glycan allergens associated with severe allergy symptoms like α -Gal. Meat extracts from pork and beef are used for commercial *in vitro* allergy diagnostics as well as equally complex mixtures of mammalian glycoproteins like bovine gelatin or pig urine proteins. These mixtures contain different amounts of α -Gal, and the exact composition is not defined (according to Phadia website and personal communication). These assays were used for several years until in 2014 a certified diagnostic singleplex analysis test based on bovine thyroglobulin (Bos d TG) as α -Gal analyte became available for routine allergy diagnostics. The aim of our study was the development of a sensitive and specific

method for the detection of anti- α -Gal-IgE. We investigated three groups of patients with two different main phenotypes (meat/innards allergy and urticaria/anaphylaxis due to unknown causes, Table 1) as well as non-meat-allergic individuals by an immunoblot with CTX and pure α -Gal coupled to human serum albumin (HSA- α -Gal) in comparison with the commercial analyte Bos d TG. The affinity of IgE from different meat allergic patients and negative controls was analyzed based on IgE cross-reactivity to the applied α -Gal-containing analytes.

Patients, Material and Methods

Patients and control individuals

This study was approved by the ethics committee (approval number 11-166) of the University of Lübeck and was conducted as a multicenter study. Patients were enrolled by 12 Dermatology Centers in Germany and the Interdisciplinary Allergy Division, Depts. of Internal Medicine of the Research Center Borstel and Lübeck University Clinic, Germany. Inclusion criteria were allergy to meat/innards, anaphylaxis and severe urticaria due to unknown causes (Tables 2 and 3). Healthy individuals with no history of meat allergy and who declared to consume meat without symptoms were included as negative controls (Table 4). IgE-mediated food allergy to meat/innards was diagnosed only if there was also a history of an acute systemic allergic reaction. Food challenge tests were not performed in all allergy centers. If performed, oral provocation tests were conducted according to open food challenge protocols, German version for physicians (13).

Immunohistochemistry on meat and kidney

Pork and beef muscle meat as well as porcine and bovine kidney tissue were fixed and paraffin-embedded by the HOPE-technique (14). Immunohistochemistry was performed as previously described (15). Briefly, after deparaffinization and blocking of endogenous peroxidase activity by 3% H₂O₂ for 10 min at room temperature, slides were washed in Tris-buffered saline (TBS) and blocking was performed for 5 min in blocking solution (Zytomed Systems). After washing in TBS, the primary antibody against the α -Gal epitope (monoclonal antibody M86, Enzo Life Sciences)

was applied in a dilution of 1:5 in antibody diluent (Zytomed Systems). After application of the blocking solution (1:2 dilution in antibody diluent) for 15 min, slides were washed in TBS (3 x 5 min). After incubation with the secondary detection antibody anti-mouse IgM/IgG-HRP (Jackson ImmunoResearch), tissues were incubated in horseradish peroxidase (HRP)-polymer (1:2 diluted in antibody diluent) for 20 min. (Zytomed Systems). After washing in TBS (3 x 5 min), aminoethylcarbazole was applied as a chromogen, slides were washed, counterstained with Mayer's hemalum and mounted.

Detection of allergen-specific IgE antibodies by CAP-FEIA (ImmunoCAP®)

All patient sera were investigated for IgE binding to α -Gal-containing CTX (1,16). Furthermore, specific IgE to the following commercially available test allergens were measured in order to characterize the individual sensitization patterns: bovine thyroglobulin (allergen Bos d TG), beef and pork meat extract, bovine serum albumin (BSA, allergen Bos d 6) and pig urine protein (all from Thermo Fisher Scientific / Phadia). In addition, total serum IgE (normal < 100 kU/L) and baseline serum tryptase (normal range < 11 μ g/l) were determined. Some sera were diluted in order to obtain a sufficient volume for analysis. The assays were performed on ImmunoCAP® 250 (Thermo Fisher Scientific / Phadia). The instrument threshold for a positive signal was 0.1 kU/L according to the manufacturer (Phadia). The cut-off commonly used for allergy diagnostics by clinicians is 0.35 kU/L, which is also used in our study.

***In vitro* cross-reactivity analysis of IgE against different α -Gal-containing proteins by dot blot**

An *in vitro* test system with different forms of α -Gal was subsequently developed which contains CTX (Erbitux®, Merck), HSA- α -Gal2 and HSA- α -Gal3 (Dextra) and Bos d TG (Sigma Aldrich / Merck). As negative controls, infliximab (Remicade®, MSD), human serum albumin (HSA, Sigma Aldrich / Merck) and human thyroglobulin (TG) (Sigma Aldrich / Merck) were included in the assay (Fig. 1). Infliximab does not contain an additional N-glycosylation site in its Fab region. As a

result, its α -Gal content is drastically reduced (16). These characteristics make infliximab an adequate negative control for IgE against α -Gal. Therapeutic antibodies cetuximab (CTX) and infliximab (IFX) were dialyzed against water (Slide-a-Lyzer, 20 kDa MWCO, Thermo Fisher Scientific) to remove additives and subsequently freeze-dried for storage (-80 °C). The analytes were applied on a stripe (about 8 cm height, 0.5 cm width) of nitrocellulose membrane (0.45 μ m pore size, Amersham, Thermo Fisher Scientific / GE Healthcare) in a volume of 1 μ l and a concentration of 1 μ g/ μ l. After drying, stripes were washed 3x with at least 1 ml TBST (50 mM, pH 7.4, 0.5% polysorbate/Tween® 20) for 20 min. Subsequently, incubations of membranes with antibodies or patients sera (1:20), diluted in TBST, were performed overnight at RT. Bound IgE antibodies were detected with an HRP-conjugated secondary antibody (monoclonal mouse anti-human IgE Fc, Southern Biotech) after incubation for 2 h in a dilution of 1:10,000 in TBST. HRP substrate was added according to the manufacturer's instructions (Clarity kit, Bio-Rad) and signals were visualized by a luminescence readout system (Chemidoc, Bio-Rad).

Statistical analysis

Associations between CAP-FEIA results obtained for meat allergic and idiopathic anaphylaxis patients as well as healthy controls were examined by using Pearson correlation coefficients and 95% confidence intervals (CI). Statistical analyses were performed with Graph Pad Prism 6.0 (Graph Pad Software).

Results

Characteristics of patient groups - α -Gal-associated meat allergic patients (MA), patients with suspected α -Gal-associated idiopathic anaphylaxis (IA) and non-meat allergic, healthy controls (HC)

The MA patient group consists of 33 individuals (13 females, 20 males), aged 20 to 82 years (Tables 1 and 2). Urticaria is the most commonly reported symptom (n=26/33). Further major symptoms documented in descending order are dyspnoea

(11/33), circulation disorders (11/33) and gastrointestinal symptoms (8/33). Note that symptoms may have occurred in parallel in individual patients. The interval between ingestion and symptom development ranged from minutes to 12 hours as indicated in the case report forms by the physician, with an average delay of 4 h (Table 1). In 2/33 patients, exercise was reported in close association with reactions after meat consumption. 11 patients reported a history of an allergic rhinoconjunctivitis, four of them had also allergic bronchial asthma. Six patients additionally suffered from allergy to food other than meat/innards (wheat, hazelnut, walnut, milk, cheese, kiwi fruit, apple, strawberry). 11/33 had a history of tick bites which was described as a possible sensitization route for α -Gal (1–3,11). An elevated concentration of total IgE (> 100 kU/l) was found in 22/33 meat allergy patients. The group of IA patients consists of 34 subjects (18 female and 16 male patients, aged 17 to 78 years) with urticaria/anaphylaxis due to unknown causes (Tables 1 and 3). For these patients, meat allergy should be confirmed or ruled out by the diagnostic tests developed in this study. 3/34 patients had a history of cat allergy, and one other had allergic rhinoconjunctivitis and pollen-associated food allergy. Two patients reported previous tick bites. The third group consists of 40 healthy individuals (28 females, 12 males), aged 19 to 65 years with a history of regular red meat consumption without meat-allergy-associated symptoms (Tables 1 and 4). 13/40 individuals reported immediate-type reactions to allergens other than meat. 13/40 remembered tick bites.

The severity of the allergic reaction against muscle meat/innards is associated with the α -Gal content of different tissues

19/33 meat allergy patients clinically reacted originally to the ingestion of mammalian innards, among them most often kidneys. 7/33 reacted to both, innards and muscle meat, and 14/33 reacted to red meat (without definite knowledge on a possible hypersensitivity to innards). Since the severity of the allergic reaction to meat depends on the consumed meat fraction (innards / muscle meat), the amount of α -Gal in kidney and muscle meat was analyzed by immunohistochemistry (4,19,20). Immunohistochemistry revealed that the content of α -Gal is highest in porcine and bovine kidney when compared to the respective muscle meat (Fig. 2). Positive staining in porcine kidney can be observed in the proximal tubules of the renal cortex

(Fig. 2 panel A). Muscles of pigs show only little staining (and thus low α -Gal content) (Fig. 2 C). In bovine skeletal muscle, signals are visible in the sarcolemma (Fig. 2 D), while the myofibrils in both species remain negative. Negative controls showed no signals (Fig. 2 E, example for porcine kidney, F, as an example for bovine muscle meat).

Sensitization patterns of meat allergy patients, idiopathic anaphylaxis patients, and healthy controls as determined by CAP-FEIA

Sera from meat allergy patients, patients with idiopathic anaphylaxis and non-meat-allergic controls were analyzed with different single allergens and allergen extracts (pork, beef, pig urine proteins, Bos d TG and Bos d 6 as well as the α -Gal-containing IgG antibody CTX). CAP-FEIA results show that 30/33 (CTX) and 23/26 (Bos d TG) of the red meat allergic patients are sensitized to α -Gal (Table 2 and Fig. 3). Best correlations for IgE diagnostics are found for CTX and Bos d TG (Fig. 3). ImmunoCAP® signals for IgE against Bos d TG are consistently lower than for CTX (except for serum MA12). Thus, especially low serum IgE concentrations against α -Gal could be detected with enhanced sensitivity by ImmunoCAP® discs with immobilized CTX (Table 2 and Fig. 3). Correlation analysis of CTX with pork, beef, pig urine proteins and Bos d TG was performed (Fig. 3). Bos d TG showed the highest correlation with CTX, while CTX shows the better performance for sera with low concentrations of specific IgE (Fig. 3). By ImmunoCAP® analyses with the α -Gal-associated allergens and allergen mixtures, positive results were found for almost all meat allergy patients (30/33 positive for IgE against CTX, Table 2). Of note, signals for IgE against Bos d TG were consistently lower than for IgE against CTX, except for serum MA12 (Table 2). ImmunoCAP® sensitivity for IgE against the α -Gal-containing analytes pig urine proteins, pork and beef meat extract was much lower than for CTX and Bos d TG (Table 2 and Fig. 3). Elevated total serum IgE (> 100 kU/l) was found in 20/33 MA-patients. Serum tryptase was higher than normal in 4/33 cases, however, the concentrations were still below the clinical level of manifest mastocytosis (< 20 μ g/l). Provocation tests with meat components confirmed the laboratory diagnostics for three patients (MA2, MA9 and MA19). Only patients MA8 and MA31 were negative for every α -Gal analyte and also for the non-glycosylated

meat allergen Bos d 6 (Table 2). Serum MA4 was negative for CTX, but positive for pork extract and pig urine proteins (Table 2).

In the group of idiopathic anaphylaxis patients with suspected meat allergy, specific IgE against α -Gal-associated CTX was detected in 5 sera (IA14, 24, 28, 32 and 33). This result is confirmed by positive signals for IgE against Bos d TG for 3 sera (IA14, 28 and 33). While the ImmunoCAP® analysis with Bos d TG could not be performed for serum IA24, it shows a low signal for beef extract (Table 3). Serum IA32, positive for anti-CTX-IgE, is negative for all other α -Gal analytes. In the IA patient group, total serum IgE concentration was higher than 100 kU/l in 7/34 patients. Serum tryptase concentrations were elevated in 5 patients, 4 had manifest mastocytosis.

Non-meat-allergic control individuals were not sensitized to α -Gal as shown in CAP-
FEIA, except for two individuals (HC32 and HC40, Table 4). Total serum IgE was elevated (> 100 kU/l) in 11/40 individuals. Serum tryptase concentration was higher than normal in 2 individuals, though below 20 μ g/l.

Sensitization patterns of meat allergy patients, idiopathic anaphylaxis patients, and healthy controls as determined by dot blot IgE cross-reactivity

Molecular characterization of the individual IgE responses of sera from each of the three patient groups (MA, IA, and HC) has the potential to identify α -Gal-sensitized, but so far unidentified meat-/innards-allergic patients in the IA patient group. This characterization was performed by immunoblot analyses using different α -Gal-containing analytes and controls. The carbohydrate α -Gal coupled to a non-immunogenic protein carrier (HSA- α -Gal) was used to determine the α -Gal-specificity along with the α -Gal-containing antibody CTX and the commercial ImmunoCAP® analyte Bos d TG (Fig. 1). As expected from the ImmunoCAP® analyses, a majority of MA patients has a strong IgE response against CTX (30/33), generally associated with IgE against the other α -Gal-containing analytes HSA- α -Gal and Bos d TG (Table 2). Thus, the IgE binding pattern associated with meat allergy (parallel binding to CTX, HSA- α -Gal and Bos d TG, e.g. meat allergy patients MA3, MA6, MA9, MA13, MA14, MA15, MA17, MA18, MA22, MA24, MA25, MA27-29, MA32, and the individual HC32, Table 2 and 4; Fig. 4) is now interpreted as “ α -Gal-dependent binding” and characteristic for α -Gal-associated meat allergy patients.

However, distinct sensitization patterns and/or IgE affinity to different α -Gal-containing analytes are observable as IgE binding only to CTX (MA1, 5, 10, 16, 19, 21, 26 and 33) or Bos d TG (MA4). Differences in IgE binding to HSA- α -Gal2 and HSA- α -Gal3 were also detected by dot blot: IgE binding to HSA- α -Gal2 was detected in 8 out of 19 HSA- α -Gal3-binding sera (Table 2). Two sera from MA patients which were negative in ImmunoCAP® analyses were also negative in the dot blot assay (MA8 and 31). The serum negative for CTX ImmunoCAP® but positive for pork meat extract (MA4), could be identified as positive for Bos d TG by dot blot assay (Table 2). All sera from non-meat-allergic controls were negative in the dot blot assay system, except for HC25, 32 and 40 (Table 4). Interestingly, for 8/40 HC sera, isolated IgE signals against the Bos d TG were observed (Bos d TG: HC4, 7, 15-17, 28, 30, and 39 (Table 4 and Fig. 5A). IgE from these sera did not cross-react with human TG (Fig. 5B) which is 77% identical on the amino acid level (Fig. S1). This IgE against Bos d TG is not derived from an autoimmunity background as determined by analysis of thyroid autoimmunity parameters (Fig. S2). No IgE signals for the negative control analytes human serum albumin (HSA) or bovine serum albumin (BSA, Bos d 6) were observed for any analyzed serum. Sera HC25 and MA22 were found to bind to IFX, an antibody which is similar to CTX in amino acid sequence but different in glycosylation (Fig. 1, Table 2 and 4). While IgE from serum HC25 is binding to CTX and IFX and does not bind to HSA- α -Gal and Bos d TG (Table 4), IgE from serum MA22 also binds to the latter α -Gal-containing analytes. Thus, patient MA22 seems to have a mixed sensitization profile: α -Gal dependent (CTX/HSA- α -Gal/Bos d TG) as well as IgG-peptide-dependent (CTX/IFX, HC25-like pattern).

IgE only against CTX was detected in IA patient sera IA12, 14, 21, 23, 24 and 32 (Table 3). For sera IA30 and 34, isolated IgE against Bos d TG was identified (Table 3). Most of the sera from the IA group show no IgE-binding to α -Gal analytes (Table 3 and Fig. 4). However, for sera IA28 and IA33, the full α -Gal cross-reactivity spectrum which is typical for meat allergy patients could be identified and was confirmed by oral challenge for patient IA28 (Table 3). Note that sera IA28 and IA33 were also identified as positive by ImmunoCAP® analyses with CTX and Bos d TG.

Discussion

In this multicentre investigation on meat allergy in Germany, 33 meat/innards allergic patients were included, and observations in clinical practice confirm that the prevalence is still increasing (5,17,18). ImmunoCAP® analyses of meat-allergic patients show that almost all patients (30/33) have elevated serum IgE against the α -Gal-containing antigen CTX (Table 1 and 2, Fig. 3). The anti-CTX IgE concentration correlated best with IgE against Bos d TG (Fig. 3) and less significantly with pork and beef, which is consistent with previous reports on the diagnostic test insufficiency of meat extracts confirming delayed anaphylaxis to red meat associated with α -Gal (19). Meat allergy patients sensitized to α -Gal react to innards more often and more severely than to muscle meat (4,19,20). Thus, meat allergy diagnosis in some cases either failed completely or could only be supported by skin prick testing (prick-to-prick) with components of innards. Patients MA2 and MA9 (Table 2) from our study experienced allergy symptoms after ingestion of innards, not muscle meat, an observation supported by allergologists from the southern part of Germany (17,18). In order to better understand the correlation between meat allergy and the severity of clinical symptoms to the type of meat, immunohistochemistry of porcine and bovine kidney in comparison with the respective muscle meat was performed, revealing a higher concentration of α -Gal in porcine kidney compared to muscle meat (Fig. 2). Thus it can be concluded that muscle meat from the same species may be better tolerated than innards depending on the total α -Gal concentration consumed with one meal (19,20).

In this study, we introduce an analysis method for the detection of IgE profiles associated with α -Gal via immunoblot. This method shall resolve individual sensitization profiles of meat/innards allergy patients (MA) as well as idiopathic anaphylaxis (IA) patients. Although a commercially available α -Gal ImmunoCAP® system based on Bos d TG has been provided for allergy testing during the course of our investigation (in 2014), the established immunoblot with the α -Gal-containing components CTX, HSA- α -Gal2/3 and Bos d TG provides enhanced immunological and diagnostic information regarding individual sensitization. HSA- α -Gal was included with and without the GlcNAc monosaccharide because of the previous observation that HSA- α -Gal3 has a 7-fold higher affinity to α -Gal-specific antibodies

(21). Comparison of the immunoblot-derived IgE cross-reactivity results from healthy control sera with the groups of MA and IA patients allows an identification of a typical “ α -Gal-associated IgE binding pattern”: CTX, HSA- α -Gal and Bos d TG (Fig. 4). Having identified this pattern, IgE sensitization patterns associated with α -Gal shall be detected in sera of IA-patients. This analysis is of considerable interest, in particular where this is the only evidence for a potential (delayed) anaphylaxis to meat. This meat-allergy-associated pattern was clearly determined for two IA-patients with suspected meat allergy: IA28 and IA33. For IA28, the diagnostic result already was substantiated via oral food challenge (Table 3 and Fig. 4). Of note, the one healthy control individual identified with this binding pattern (HC32) had reported regular meat consumption without any problems. Since dot test with his serum revealed the “meat allergy” sensitization pattern, it remains to be determined if a clinical reaction in this individual may develop after ingestion of a very high amount of meat/innards. Those MA patients negative for all parameters in both singleplex and dot blot analysis, but with clinical reactions to meat, may be IgE-reactive to meat allergens not available for allergy diagnostic tests so far (myoglobulin, hemoglobin, the 81 kDa-protein, the 51 kDa-phosphoglucosylase) (8). Sera IA12, 21, 30 and 34 were negative in ImmunoCAP® with CTX but positive in the dot blot assay for CTX (IA12 and 21) or Bos d TG (IA30 and 34) demonstrating individual differences regarding the sensitization to the α -Gal-containing analytes. Interestingly, by serum IgE cross-reactivity analysis, we could demonstrate that isolated IgE against Bos d TG without HSA- α -Gal and CTX association seems to be relatively frequent in the (healthy) population. However, IgE binding to bovine TG is either dependent on glycans different from the glycans present on CTX and HSA- α -Gal or additionally dependent on other epitopes which are only part of bovine (and not human) TG as no binding was observed to human TG which is 77% identical on the amino acid level (Fig. 5 and S1) (22,23,24). This IgE is most likely not derived from an autoimmune background as confirmed by full thyroid autoimmune serum diagnostics (Fig. 5 and S2). It is very important to mention that TG as a natural bovine protein is not defined in its glycan structure and contains not only α -Gal, but also the immunogenic non-human carbohydrate N-glycolyl neuraminic acid (Neu5Gc) and the high mannose N-glycan motif which may contribute to IgE-binding (Fig. 1). Although it has been published that the monomer Neu5Gc bound to a hydrocarbon linker is not IgE-binding (25), natural Neu5Gc as part of an N-glycan attached to an allergen

is structurally different and may be binding to IgE. It has been shown that Neu5Gc is highly immunogenic so that it can be used to arm the human immune system for cancer treatment (26). The origin of the signals detected in the dot blot assay for IgE against Bos d TG without apparent α -Gal cross-reactivity remains to be determined.

Taken together, in our study we show individual differences between the sensitization profiles of MA and IA-patients to different α -Gal-containing analytes. Whereas MA patients with a strong history of an α -Gal syndrome often can be confirmed *in vitro* by the commercially available singleplex ImmunoCAP® analysis, diagnostics of patients with idiopathic anaphylaxis and suspected meat allergy is more challenging. Having identified the IgE binding pattern associated with meat allergy patients in dot blot, thorough analysis of sera from the IA patient group may lead to the identification of risk-associated IgE binding patterns via dot blot, which is already evaluated in many more IA patients attending our allergy outpatient clinic. By a combination of different *in vitro* analysis techniques for the detection and characterization of IgE against alpha-Gal, such as the introduced dot blot assay system, a more differentiated ImmunoCAP diagnostic as well as subsequent provocation tests, hopefully many more previously underdiagnosed patients with idiopathic anaphylaxis can be diagnosed correctly. We could show that the CTX ImmunoCAP® provides enhanced sensitivity for some patients with low serum concentration of IgE against α -Gal. Thus, it may be concluded that especially these patients (from the IA patient group) benefit from an additional analysis by ImmunoCAP® with CTX.

Acknowledgments

We would like to thank Daniela Warneke, Lea Bendler and Lisa Grigoleit from the Research Center Borstel for excellent technical assistance. The authors are very grateful for the quick and thorough ImmunoCAP® analyses performed by Angela Willanzheimer at IPM Biotech and Labor Lademannbogen, Hamburg, Germany. The authors are grateful for the kind support by Dr. Andreas Lämmel, Head of the Medical Laboratory.

Authors` contributions

UJ designed the study, selected and included patients, analyzed the data and wrote the manuscript. AH designed and performed experiments, analyzed the data and wrote the manuscript. SM designed and performed experiments and recruited healthy meat consumers as controls. AK performed ImmunoCAP® experiments and analyzed the data. TG designed and performed immunohistochemistry experiments. BK, AL, BP, AW, RV, JF, TB, WA, FR, HM, NW, RT, MW, IW, JS provided sera from meat allergy patients and idiopathic anaphylaxis patients. WMB performed preliminary immunoblots with different meat/innards extracts. TPM provided CTX ImmunoCAP® discs and reviewed and corrected the manuscript. All authors read and approved the manuscript.

Conflict of Interest

UJ, SM, BK, BP, AW, RV, PS, TB, WA, FR, HM, NW, RT, MW, IW, JS, WMB, TAP and AH declare no conflicts of interest.

Funding

Parts of the study were supported by the the German Ministry for Economy and Energy (BMWi) via the AiF ZIM program, grant no KF 2784701AJ0.

Tables and Figures

Table 1 Characteristics of the analyzed groups meat allergy patients, idiopathic anaphylaxis patients and healthy controls. For a detailed description of the groups see section “Patients, Materials and Methods”.

Table 2 Serum IgE analyses of meat/innards allergy patients (n=33) by ImmunoCAP® and dot blot. n.p., not performed (due to lack of serum).

Table 3 Serum IgE analyses of idiopathic anaphylaxis patients (n=34) by ImmunoCAP® and dot blot. n.p. not performed (due to lack of serum).

Table 4 Serum IgE analyses of non-meat allergic healthy controls (n=40) by ImmunoCAP® and dot blot. n.p., not performed, allergy status: A, known allergy other than meat allergy.

Figure 1 Glycan characteristics of α -Gal analytes used in this study. Note that CTX and IFX differ in their glycan content but are relatively similar on the amino acid level, even in their variable Fab regions (confirmed by sequence alignment). CTX and Bos d TG also contain the immunogenic glycan Neu5Gc as well as branches of the N-glycan high mannose motif (16,22,23). The α -Gal and sialic acid content was determined by lectin binding assay with lectins from *Bandeiraea simplicifolia*, BS-I (27) and *Sambucus nigra* agglutinin, SNA (Vector Labs).

Figure 2 Immunohistochemical detection of α -Gal. Using a monoclonal antibody and a polymer-detection-system with aminoethylcarbazole as a chromogen (red-brown), α -Gal was detected in kidney of pork (A) and beef (B). Positive staining in pork can be observed in the proximal tubules of the renal cortex (A). Muscles of pork show only occasionally a low level of staining (C). In bovine skeletal muscle, signals are visible in the sarcolemma (D), while the myofibrils in both species remain negative. Negative controls without primary antibody showed no signals (E as an example for pork kidney, F as an example for a bovine skeletal muscle). All magnifications are 400x.

Figure 3 Meat allergy diagnostics and association with α -Gal-containing analytes. Serum IgE against α -Gal from MA patients was detected by ImmunoCAP® diagnostics using α -Gal (CTX), pork and beef extract, pig urine proteins (PUP) and bovine thyroglobulin (Bos d TG). No association was found with the non-glycosylated meat allergen BSA (Bos d 6). IgE against CTX correlates best with Bos d TG ($R^2=0.77$). CTX ImmunoCAP® is more sensitive than the commercial ImmunoCAP with Bos d TG when serum IgE concentration is low (bottom right plot, axis adjusted).

Figure 4 Binding pattern of serum IgE from patient groups MA (meat allergy patients) and IA (idiopathic anaphylaxis patients). Analysis of the specificity of IgE against α -Gal using different α -Gal-containing analytes (cetuximab, CTX, HSA- α -Gal and bovine thyroglobulin, Bos d TG). The common, most prominent binding pattern is detected against the α -Gal-containing analytes CTX/HSA- α -Gal/Bos d TG. Additionally, isolated binding against CTX (MA2 and IA32) and Bos d TG (MA28, IA30 and IA34) was observed. Note that among the IA patients, IA28 and IA33 show a characteristic MA pattern, which is clinically supported by provocation for IA28. The development time for MA patients (top) was 1 s, for the IA patients (bottom figure) 60 s.

Figure 5 Healthy Control sera contain IgE against Bos d TG (commercial α -Gal ImmunoCAP® analyte bovine thyroglobulin) not associated with other α -Gal analytes and not cross-reactive with human TG. One α -Gal-associated IgE binding pattern was identified (HC32). Additionally, one pattern resembling non- α -Gal dependent IgE binding to CTX and IFX was detected (HC25) as well as isolated IgE against bovine TG (HC4, 7, 15, 16, 17, 28, 30, and 39). HC28 and HC29 show weak IgE binding to CTX only (HC29) or CTX associated with Bos d TG but not with HSA- α -Gal (HC28). n.p., not performed (due to lack of serum).

Supplementary Figures

Figure S1 Amino acid sequence alignment of bovine thyroglobulin (Bos d TG, “Sbjct”) and human thyroglobulin (“Query”). The alignment indicates 77% identical amino acids.

Figure S2 Thyroid autoimmune diagnostics of sera which are positive for IgE against bovine thyroglobulin (Bos d TG). For IgE serum results see Table 4 and Figure 5.

References

1. Chung CH, Mirakhur B, Chan E, Le Q-T, Berlin J, Morse M et al. Cetuximab-Induced Anaphylaxis and IgE Specific for Galactose- α 1,3-Galactose. *N Engl J Med* 2008;**358**:1109–1117.
2. 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- α 1,3-galactose. *J Allergy Clin Immunol* 2009;**123**:426–433.e2.
3. 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**:510–511.
4. Fischer J, Yazdi AS, Biedermann T. Clinical spectrum of α -Gal syndrome: from immediate-type to delayed immediate-type reactions to mammalian innards and meat. *Allergo J Int* 2016;**25**:55–62.
5. Apostolovic D, Tran TAT, Starkhammar M, Sánchez-Vidaurre S, Hamsten C, Van Hage M. The red meat allergy syndrome in Sweden. *Allergo J Int* 2016;**25**:49–54.
6. Villalta D, Pantarotto L, Da Re M, Conte M, Sjolander S, Borres MP et al. High prevalence of sIgE to Galactose- α 1,3-galactose in rural pre-Alps area: a cross-sectional study. *Clin Exp Allergy* 2016;**46**:377-380.
7. Kollmann D, Nagl B, Ebner C, Emminger W, Wöhrl S, Kitzmüller C et al. The quantity and quality of α -gal-specific antibodies differ in individuals with and without delayed red meat allergy. *Allergy* 2017;**72**:266-273.
8. Jappe U. Update on meat allergy. alpha-Gal: a new epitope, a new entity? *Hautarzt* 2012;**63**:299–306.

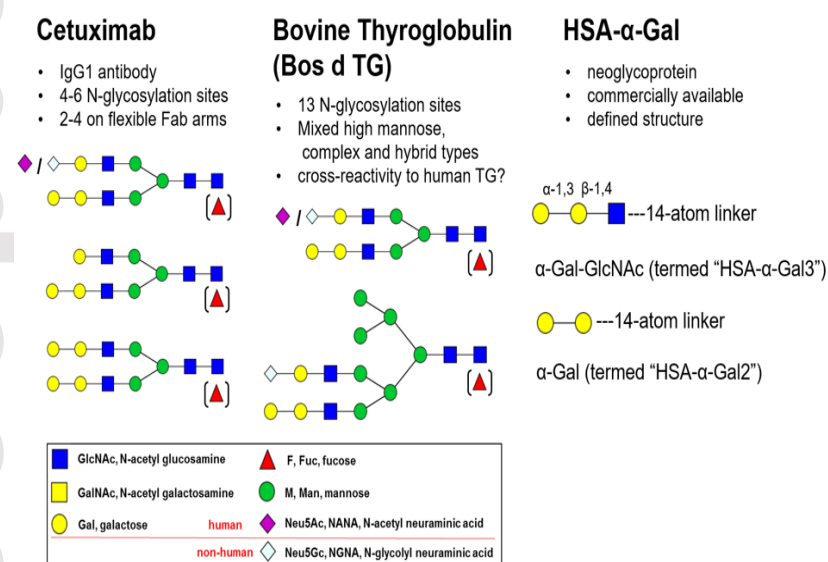
9. Jappe U, Platts-Mills T, Przybilla B, Kreft B, Ludwig A, Walker A et al. IgE-reactivity to galactose- α -1,3-galactose: a prospective multicentre study on meat allergy, urticaria and anaphylaxis. *Allergy* 2012;**67**, Suppl. 96:96.
10. Commins SP, James HR, Kelly LA, Pochan SL, Workman LJ, Perzanowski 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**:1286–1293.e6.
11. Hamsten C, Starkhammar M, Tran T a. T, 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**:549–552.
12. Michel S, Scherer K, Heijnen IAFM, Bircher AJ. Skin prick test and basophil reactivity to cetuximab in patients with IgE to α -gal and allergy to red meat. *Allergy* 2014;**69**:403–405.
13. Niggemann B, Beyer K, Erdmann S, Fuchs T, Kleine-Tebbe J, Lepp U et al. Standardisierung von oralen Provokationstests bei Verdacht auf Nahrungsmittelallergie. *Allergo J* 2011;**20**:149–60.
14. Olert J, Wiedorn KH, Goldmann T, Kühl H, Mehraein Y, Scherthan H et al. HOPE fixation: a novel fixing method and paraffin-embedding technique for human soft tissues. *Pathol Res Pract* 2001;**197**:823–826.
15. Marwitz S, Abdullah M, Vock C, Fine JS, Visvanathan S, Gaede KI et al. HOPE-BAL. *J Histochem Cytochem* 2011;**59**:601–614.
16. Ayoub D, Jabs W, Resemann A, Evers W, Evans C, Main L et al. Correct primary structure assessment and extensive glyco-profiling of cetuximab by a combination of intact, middle-up, middle-down and bottom-up ESI and MALDI mass spectrometry techniques. *mAbs* 2013;**5**:699–710.
17. Biedermann T, Fischer J, Yazdi A. Mammalian meat allergy: a diagnostic challenge. *Allergo J Int* 2015;**24**:81–83.
18. Fischer J, Hebsaker J, Caponetto P, Platts-Mills TAE, Biedermann T. Galactose- α -1,3-galactose sensitization is a prerequisite for pork-kidney allergy and cofactor-related mammalian meat anaphylaxis. *J Allergy Clin Immunol* 2014;**134**:755–759.e1.
19. 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- α -1,3-galactose. *Allergy* 2012;**67**:699–704.
20. Hilger C, Fischer J, Swiontek K, Hentges F, Lehnert C, Eberlein B et al. Two galactose- α -1,3-galactose carrying peptidases from pork kidney mediate anaphylactogenic responses in delayed meat allergy. *Allergy* 2016;**71**:711–719.
21. Rispens T, Derksen NIL, Commins SP, Platts-Mills TA, Aalberse RC. IgE Production to α -Gal Is Accompanied by Elevated Levels of Specific IgG1 Antibodies and Low Amounts of IgE to Blood Group B. *PLoS ONE* 2013;**8**:e55566.
22. Rawitch AB, Pollock HG, Yang SX. Thyroglobulin glycosylation: location and nature of the N-linked oligosaccharide units in bovine thyroglobulin. *Arch Biochem Biophys* 1993;**300**:271–279.

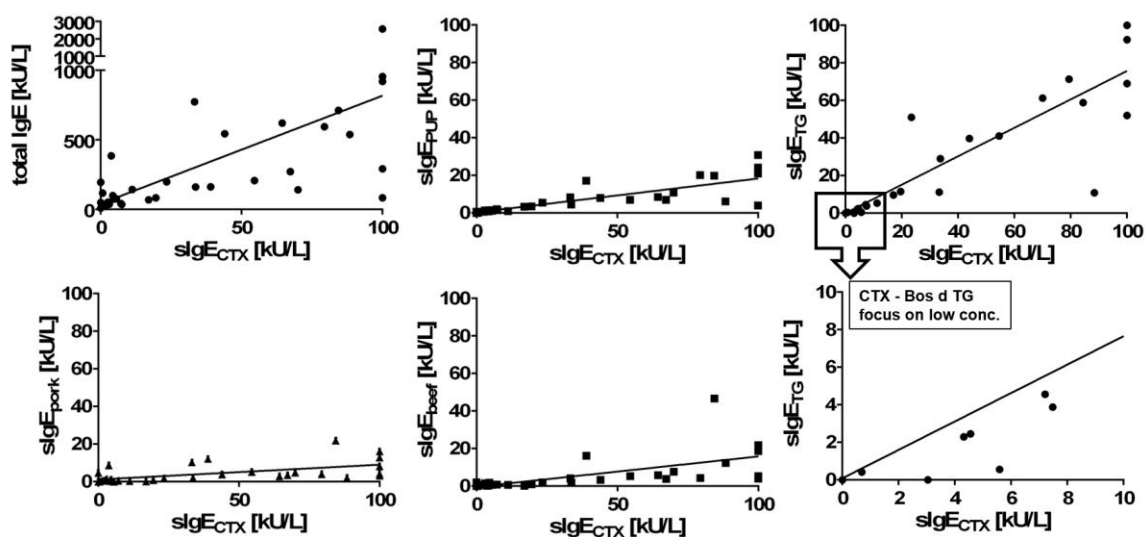
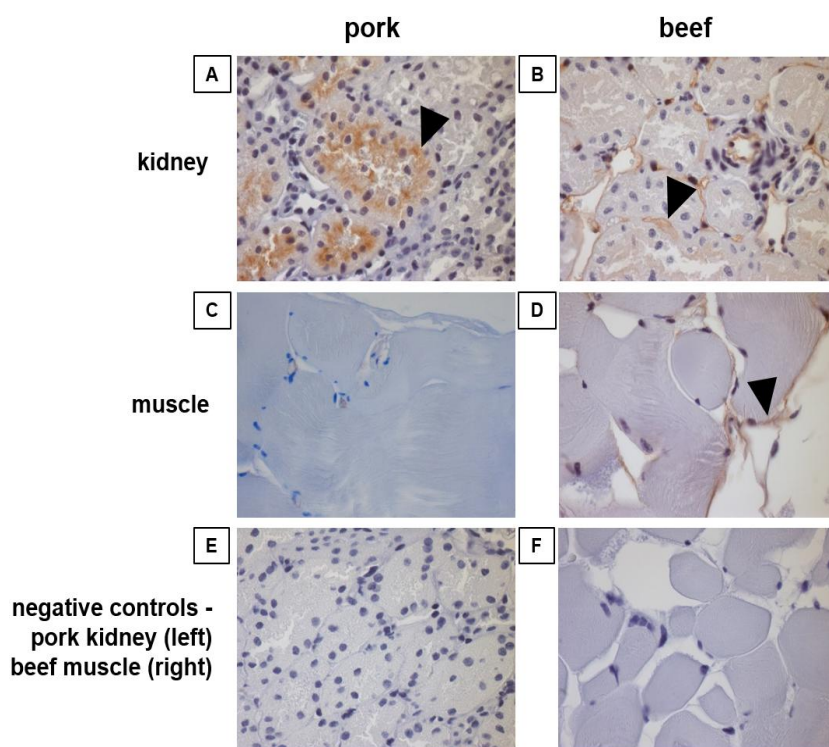
23. Yang S-X, Pollock HG, Rawitch AB. Glycosylation in Human Thyroglobulin: Location of the N-Linked Oligosaccharide Units and Comparison with Bovine Thyroglobulin. *Arch Biochem Biophys* 1996;**327**:61–70.
24. Kohno Y, Nakajima H, Tarutani O. Interspecies cross-reactive determinants of thyroglobulin recognized by autoantibodies. *Clin Exp Immunol* 1985;**61**:44.
25. Apostolovic D, Tran T a. T, Sánchez-Vidaurre S, Cirkovic Velickovic T, Starkhammar M, Hamsten C et al. Red meat allergic patients have a selective IgE response to the α -Gal glycan. *Allergy* 2015;**70**:1497-1500.
26. Pearce OMT, Läubli H. Sialic acids in cancer biology and immunity. *Glycobiology* 2016;**26**:111–128.
27. Kirkeby S, Moe D. Binding of Griffonia simplicifolia 1 isolectin B4 (GS1 B4) to α -galactose antigens. *Immunol Cell Biol* 2001;**79**:121–127.

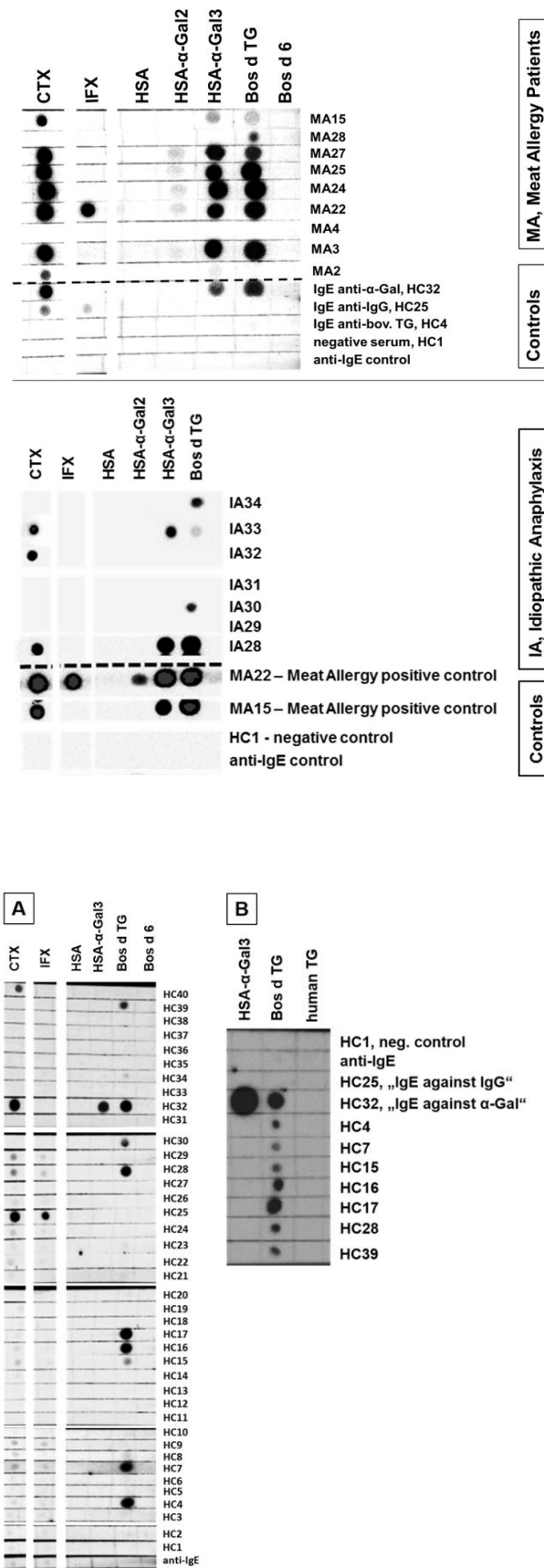
A) Functional glycan content determined by lectin binding analysis

Analyte	Glycan content	
	α -Gal	Sialic Acid
Cetuximab	↑ high	↑ high
Infliximab	↓ (very low)	↓ low
HSA- α -Gal	↑ high	↓ not detect.
Bov. Thyroglobulin	↑ high	↑ high

B) Characteristics and predominant N-glycans of α -Gal-containing analytes







[illegible]

