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Meat allergy associated with galactosyl- α -(1,3)-galactose (α -Gal) —Closing diagnostic gaps by anti- α -Gal IgE immune profiling

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AUTHOR 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, and JS provided sera from MA patients and IA patients. WMB performed preliminary immunoblots with different meat/innards extracts. TPM provided CTX ImmunoCAP® disks and reviewed and corrected the manuscript. All authors read and approved the manuscript.

CONFLICTS OF INTEREST

The other authors declare no conflict of interest.

SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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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 (CTX) and is associated with delayed anaphylaxis to red meat. In this study, different α -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 MA, 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 CTX, bovine thyroglobulin (Bos d TG), and human serum albumin (HSA)-conjugated α -Gal.

Results: Singleplex allergy diagnostics using the α -Gal analytes CTX and Bos d TG confirms the history of MA 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 CTX, Bos d TG, and HSA- α -Gal) was identified, which is associated with MA.

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.

Keywords

alpha-Gal; bovine thyroglobulin; cetuximab; delayed anaphylaxis; meat allergy

1 | 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 (MA) 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 MA 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 MA and IgE to α -Gal was discovered, MA was diagnosed rarely, and only few studies and case reports were published describing the nonglycosylated 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, MA 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.

2 | PATIENTS, MATERIAL AND METHODS

2.1 | 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 multi-center study. Patients were enrolled by 12 Dermatology Centers in Germany and the Interdisciplinary Allergy Division, Departments 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 MA 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

2.2 | Immunohistochemistry on meat and kidney

Pork and beef muscle meat as well as porcine and bovine kidney tis-sue 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 minutes at room temperature, slides were washed in Tris-buffered saline (TBS) and blocking was performed for 5 minutes 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 minutes, slides were washed in TBS (3×5 minutes). 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 minutes. (Zytomed Systems). After washing in TBS (3×5 minutes), aminoethylcarbazole was applied as a chromogen and slides were washed, counterstained with Mayer's hemalum, and mounted.

2.3 | 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, BSA (allergen Bos d 6), and pig urine protein (all from Thermo Fisher Scientific/Phadia AB, Uppsala, Sweden). 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 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 cutoff commonly used for allergy diagnostics by clinicians is 0.35 kU/L, which is also used in our study.

2.4 | 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 (Erbix®; Merck, Darmstadt, Germany), HSA- α -Gal2 and HSA- α -Gal3 (Dextra, Reading, UK), and Bos d TG (Sigma-Aldrich, Munich, Germany/Merck). As negative controls, infliximab (IFX; Remicade®; MSD), HSA (Sigma-Aldrich/Merck), and human thyroglobulin (TG; Sigma-Aldrich/Merck) were included in the assay (Figure 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 IFX an adequate negative control for IgE against α -Gal. Therapeutic antibodies CTX and 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, Freiburg, Germany) in a volume of 1 μ L and a concentration of 1 μ g/ μ L. After drying, stripes were washed 3× with at least 1 mL TBST (50 mmol/L, pH 7.4, 0.5% polysorbate/Tween®-20) for 20 minutes. 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, Birmingham, AL, USA) after incubation for 2 hours in a dilution of 1:10 000 in TBST. HRP substrate was added according to the manufacturer's instructions (Clarity kit; Bio-Rad, Munich, Germany) and signals were visualized by a luminescence readout system (Chemidoc; Bio-Rad).

2.5 | Statistical analysis

Associations between CAP-FEIA results obtained for meat-allergic and idiopathic anaphylaxis (IA) patients as well as healthy controls (HCs) were examined using Pearson correlation coefficients and 95% confidence intervals (CI). Statistical analyses were performed with GraphPad Prism 6.0 (GraphPad Software, San Diego, CA, USA).

3 | RESULTS

3.1 | Characteristics of patient groups— α -Gal-associated meat-allergic patients (MA), patients with suspected α -Gal-associated IA, and non-meat-allergic, HCs

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 hours (Table 1). In 2 of 33 patients, exercise was reported in close association with reactions after meat consumption. Eleven patients reported a history of an allergic rhinoconjunctivitis and 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). Eleven of 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 of 33 MA patients. The group of IA patients consists of 34 subjects (18 female and 16 male patients, aged 17–78 years) with urticaria/anaphylaxis due to unknown causes (Tables 1 and 3). For these patients, MA should be confirmed or ruled out by the diagnostic tests developed in this study. Three of 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 and 12 males), aged 19–65 years with a history of regular red meat consumption without MA-associated symptoms (Tables 1 and 4). Thirteen of 40 individuals reported immediate-type reactions to allergens other than meat. Thirteen of 40 remembered tick bites.

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

Nineteen of 33 MA patients clinically reacted originally to the ingestion of mammalian innards, among them most often kidneys. Seven of 33 reacted to both, innards and muscle meat, and 14 of 33 reacted to red meat (without definite knowledge on a possible hypersensitivity to innards). As 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.^{4,17,18} Immunohistochemistry revealed that the content of α -Gal is highest in porcine and bovine kidney when compared to the respective muscle meat (Figure 2). Positive staining in porcine kidney can be observed in the proximal tubules of the renal cortex (Figure 2 panel A). Muscles of pigs show only little staining (and thus low α -Gal content; Figure 2C). In bovine skeletal muscle, signals are visible in the

sarcolemma (Figure 2D), while the myofibrils in both species remain negative. Negative controls showed no signals (Figure 2E, example for porcine kidney, F, as an example for bovine muscle meat).

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

Sera from MA patients, patients with IA, 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 of 33 (CTX) and 23 of 26 (Bos d TG) of the red meat-allergic patients are sensitized to α -Gal (Table 2 and Figure 3). Best correlations for IgE diagnostics are found for CTX and Bos d TG (Figure 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® disks with immobilized CTX (Table 2 and Figure 3). Correlation analysis of CTX with pork, beef, pig urine proteins, and Bos d TG was performed (Figure 3). Bos d TG showed the highest correlation with CTX, while CTX shows the better performance for sera with low concentrations of specific IgE (Figure 3). By ImmunoCAP® analyses with the α -Gal-associated allergens and allergen mixtures, positive results were found for almost all MA 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 and pork and beef meat extract was much lower than for CTX and Bos d TG (Table 2 and Figure 3). Elevated total serum IgE (>100 kU/L) was found in 20 of 33 MA patients. Serum tryptase was higher than normal in four of 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 nonglycosylated 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 IA patients with suspected MA, specific IgE against α -Gal-associated CTX was detected in five sera (IA14, 24, 28, 32, and 33). This result is confirmed by positive signals for IgE against Bos d TG for three 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 seven of 34 patients. Serum tryptase concentrations were elevated in five patients, and four 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 of 40 individuals. Serum tryptase concentration was higher than normal in two individuals, though below 20 μ g/L.

3.4 | 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 nonimmunogenic 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 (Figure 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 MA (parallel binding to CTX, HSA- α -Gal, and Bos d TG, eg, MA patients MA3, MA6, MA9, MA13, MA14, MA15, MA17, MA18, MA22, MA24, MA25, MA27–29, MA32, and the individual HC32, Tables 2 and 4; Figure 4) is now interpreted as “ α -Gal-dependent binding” and characteristic for α -Gal-associated MA 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 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 of 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 (Figure 5B) which is 77% identical on the amino acid level (Figure S1). This IgE against Bos d TG is not derived from an autoimmunity background as determined by analysis of thyroid autoimmunity parameters (Figure S2). No IgE signals for the negative control analytes HSA or 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 (Figure 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 Figure 4). However, for sera IA28 and IA33, the full α -Gal cross-reactivity spectrum which is typical for MA 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.

4 | DISCUSSION

In this multicentre investigation on MA in Germany, 33 meat-/innards-allergic patients were included, and observations in clinical practice confirm that the prevalence is still increasing.^{5,19,20} ImmunoCAP® analyses of meat-allergic patients show that almost all patients (30/33) have elevated serum IgE against the α -Gal-containing antigen CTX (Tables 1 and 2, Figure 3). The anti-CTX IgE concentration correlated best with IgE against Bos d TG (Figure 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.¹⁷ Meat allergy patients sensitized to α -Gal react to innards more often and more severely than to muscle meat.^{4,17,18} Thus, MA 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.^{19,20} In order to better understand the correlation between MA 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 (Figure 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.^{17,18}

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 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 sevenfold higher affinity to α -Gal-specific antibodies.²¹ Comparison of the immunoblot-derived IgE cross-reactivity results from HC 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 (Figure 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 MA-associated pattern was clearly determined for two IA patients with suspected MA: IA28 and IA33. For IA28, the diagnostic result already was substantiated via oral food challenge (Table 3 and Figure 4). Of note, the one HC individual identified with this binding pattern (HC32) had reported regular meat consumption without any problems. As dot test with his serum revealed the “MA sensitization pattern”, it remains to be determined whether 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 (myoglobin, hemoglobin, the 81-kDa protein, the 51-kDa phosphoglucomutase).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 (Figures 5 and S1).^{22–24} This IgE is most likely not derived from an autoimmune background as confirmed by full thyroid autoimmune serum diagnostics (Figures 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 nonhuman carbohydrate N-glycolyl neuraminic acid (Neu5Gc) and the high mannose N-glycan motif which may contribute to IgE binding (Figure 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 IA and suspected MA is more challenging. Having identified the IgE binding pattern associated with MA 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 α -Gal, such as the introduced dot blot assay system, a more differentiated ImmunoCAP® diagnostic as well as subsequent provocation tests, many more previously underdiagnosed patients with IA 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations:

Bos d TG	<i>Bos domesticus</i> (bovine) thyroglobulin
BSA	bovine serum albumin
CTX	cetuximab
HC	healthy control
HRP	horseradish peroxidase
HSA	human serum albumin
IA	idiopathic anaphylaxis
IFX	infliximab
MA	meat allergy
Neu5Gc	N-glycolyl neuraminic acid
TBS	Tris-buffered saline
α-Gal	galactosyl-α-(1,3)-galactose, for details see also Figure 1

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(A) Functional glycan content determined by lectin binding analysis

	Glycan content	
Analyte	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

FIGURE 1.

Glycan characteristics of α -Gal analytes used in this study. Note that cetuximab (CTX) and infliximab 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 domesticus* (bovine) thyroglobulin 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, Peterborough, UK)

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 400 $\times$

FIGURE 3.

Binding pattern of serum IgE from patient groups meat allergy (MA patients) and idiopathic anaphylaxis (IA patients). Analysis of the specificity of IgE against α -Gal using different α -Gal-containing analytes (cetuximab, CTX, human serum albumin [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 and for the IA patients (bottom figure) was 60 s

FIGURE 4.

Meat allergy (MA) diagnostics and association with α-Gal-containing analytes. Serum IgE against α-Gal from MA patients was detected by ImmunoCAP® diagnostics using α-Gal (cetuximab [CTX]), pork and beef extract, pig urine proteins (PUP), and bovine thyroglobulin (Bos domesticus (bovine) thyroglobulin [Bos d TG]). No association was found with the nonglycosylated meat allergen bovine serum albumin (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)

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FIGURE 5.

Healthy control sera contain IgE against *Bos domesticus* (bovine) thyroglobulin (commercial α-Gal ImmunoCAP® analyte bovine thyroglobulin) not associated with other α-Gal analytes (A) and not cross-reactive with human TG (B). One α-Gal-associated IgE binding pattern was identified (HC32). Additionally, one pattern resembling non-α-Gal-dependent IgE binding to cetuximab (CTX) and infliximab (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).

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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”

	MA, meat allergy patients <i>n</i> = 33	IA, idiopathic anaphylaxis patients <i>n</i> = 34	HC, healthy control individuals <i>n</i> = 40
Gender, % female	36	56	70
Mean age (y)	54	44	38
Average serum IgE (kU/L)	341	147	83
Positive for IgE against α -Gal (ImmunoCAP®)	30/33 (CTX) 23/26 (Bos d TG)	3/34 (CTX) 3/14 (Bos d TG)	2/16 (CTX) 2/40 (Bos d TG)
Trigger allergen			
Muscle meat	14/33	26/34	/
Innards (including sausage)	19/33	2/34	/
Allergy symptoms			
Urticaria	25/33	17/34	/
Dyspnoe	11/33	10/34	/
Gastrointestinal disorder	8/33	8/34	/
Angioedema	8/33	15/34	/
Circulation disorder	11/33	7/34	/
Average time to symptoms (range: several min to 12 h)	4.0 h	4.5 h	/

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) IgE (kU/L) ImmunoCAP

IgE (kU/L) ImmunoCAP®										
ID	CTX	Bos d TG	Pork	Beef	Pig urine proteins	Bos d 6	Total IgE	Tryptase (µg/L)	Provocation	IgE pattern (dot blot)
MA1	11.20	5.36	0.33	0.59	1.05	<0.1	144.00	1.40	Innards neg./meat neg.	CTX
MA2	7.21	4.56	0.47	0.78	2.07	<0.1	42.20	2.71	Innards pos./meat neg.	CTX/HSA-α-Gal3
MA3	>100	69.00	8.18	18.60	30.80	0.60	2574.00	5.60		CTX/HSA-α-Gal2 + 3/Bos d TG
MA4	<0.1	n.p.	4.70	<0.1	0.66	<0.1	195.00	2.50		Bos d TG
MA5	3.04	<0.1	1.18	1.58	1.32	<0.1	38.20	15.90		CTX
MA6	>100	92.40	3.14	5.28	4.06	<0.1	83.90	2.80		CTX/HSA-α-Gal2 + 3/Bos d TG
MA7	3.73	n.p.	8.67	0.15	1.41	<0.1	386.00	4.50		CTX/Bos d TG
MA8	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	49.80	1.70		Negative
MA9	>100	52.02	4.22	4.04	n.p.	n.p.	292.00	6.40	Kidney pos./pork meat neg.	CTX/HSA-α-Gal3/Bos d TG
MA10	4.32	2.30	0.30	0.40	0.80	<0.1	100.00	7.20		CTX
MA11	17.00	9.72	0.10	0.10	3.40	0.11	69.00	8.54		CTX/HSA-α-Gal3
MA12	23.40	51.00	1.80	2.02	5.54	<0.1	199.00	2.19		CTX/HSA-α-Gal3
MA13	>100	>100	12.90	21.80	24.10	0.5	921.00	3.30		CTX/HSA-α-Gal2 + 3/Bos d TG
MA14	19.60	11.50	0.89	1.00	3.47	<0.1	84.30	1.90		CTX/HSA-α-Gal3/Bos d TG
MA15	88.40	10.90	2.00	12.30	6.16	<0.1	539.00	16.50		CTX/HSA-α-Gal2 + 3/Bos d TG
MA16	0.69	0.42	0.13	0.11	0.17	<0.1	118.00	3.00		CTX
MA17	33.70	29.00	2.00	1.88	4.53	0.12	162.00	6.80		CTX/HSA-α-Gal3/Bos d TG
MA18	54.60	41.10	5.19	5.26	6.96	0.12	208.00	4.96		CTX/HSA-α-Gal3 Bos d TG
MA19	5.59	0.56	<0.1	0.60	1.24	0.15	76.00	1.30	Innards pos./meat pos.	CTX
MA20	33.30	11.22	10.30	4.10	8.26	0.28	774.00	6.50		CTX/HSA-α-Gal3
MA21	1.73	n.p.	0.42	0.64	0.60	<0.1	23.80	17.30		CTX
MA22	44.00	39.78	3.86	3.25	7.99	0.17	544.00	2.80		CTX/IFX/HSA-α-Gal2 + 3/Bos d TG
MA23	2.56	n.p.	0.59	0.64	0.71	<0.1	52.70	8.40		n.p.
MA24	79.40	71.40	4.00	4.30	20.20	0.62	595.00	14.20		CTX/HSA-α-Gal2 + 3/Bos d TG
MA25	84.40	58.86	21.90	46.60	19.90	0.92	712.00	2.00		CTX/HSA-α-Gal2 + 3/Bos d TG
MA26	4.56	2.45	1.00	1.80	1.43	<0.1	74.50	5.20		CTX

IgE (kU/L) ImmunoCAP®										
ID	CTX	Bos d TG	Pork	Beef	Pig urine proteins	Bos d 6	Total IgE	Tryptase (µg/L)	Provocation	IgE pattern (dot blot)
MA27	>100	>100	16.10	21.00	21.00	0.42	955.00	7.20		CTX/HSA-α-Gal2 + 3/Bos d TG
MA28	39.00	n.p.	12.10	16.20	17.20	0.87	163.00	2.39		CTX/HSA-α-Gal3/Bos d TG
MA29	70.00	61.30	4.77	7.65	10.80	0.1	141.00	6.80		CTX/HSA-α-Gal3/Bos d TG
MA30	64.40	n.p.	2.86	5.91	8.53	<0.1	621.00	10.30		n.p.
MA31	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	9.90	6.80		Negative
MA32	7.48	3.88	n.p.	n.p.	1.45	0.29	36.80	4.50		CTX/HSA-α-Gal3/Bos d TG
MA33	67.30	n.p.	3.44	3.88	6.94	<0.1	272.00	<5.0		CTX

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) IgE (kU/L)

ImmunoCAP®										
ID	CTX	Bos d TG	Pork	Beef	Pig urine proteins	Bos d 6	Total IgE	Tryptase (µg/L)	Provocation	IgE pattern (dot blot)
IA1	<0.1	<0.1	<0.1	<0.1	n.p.	n.p.	17.70	4.37		Negative
IA2	<0.1	n.p.	<0.1	<0.1	<0.1	<0.1	84.00	2.85		Negative
IA3	<0.1	n.p.	<0.1	<0.1	<0.1	<0.1	180.00	4.68		Negative
IA4	<0.1	n.p.	<0.1	<0.1	<0.1	<0.1	184.00	9.71		Negative
IA5	<0.1	n.p.	<0.1	<0.1	<0.1	<0.1	165.00	5.80		Negative
IA6	<0.1	n.p.	n.p.	n.p.	n.p.	n.p.	n.p.	3.35		Negative
IA7	<0.1	n.p.	<0.1	<0.1	<0.1	<0.1	57.30	2.96		Negative
IA8	<0.1	n.p.	<0.1	<0.1	<0.1	<0.1	114.00	4.39		Negative
IA9	<0.1	n.p.	<0.1	<0.1	<0.1	<0.1	9.60	4.76		Negative
IA10	<0.1	n.p.	<0.1	<0.1	<0.1	<0.1	35.90	6.32		Negative
IA11	<0.1	n.p.	<0.1	<0.1	<0.1	<0.1	38.10	5.59		Negative
IA12	<0.1	n.p.	<0.1	<0.1	<0.1	<0.1	771.00	4.26		CTX
IA13	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	83.90	1.09		Negative
IA14	3.01	0.82	0.51	0.54	n.p.	n.p.	20.90	33.80	Pork meat neg.	CTX/HSA-α-Gal3
IA15	<0.1	n.p.	<0.1	<0.1	<0.1	<0.1	9.20	31.80		Negative
IA16	<0.1	n.p.	<0.1	<0.1	<0.1	<0.1	58.20	4.19		Negative
IA17	<0.1	<0.1	<0.1	<0.1	n.p.	n.p.	n.p.	22.70	Pork meat pos.	Negative
IA18	<0.1	n.p.	<0.1	<0.1	<0.1	<0.1	11.60	14.70		Negative
IA19	<0.1	n.p.	<0.1	<0.1	<0.1	<0.1	4.00	2.80		Negative
IA20	<0.1	n.p.	<0.1	<0.1	<0.1	<0.1	20.80	3.59		Negative
IA21	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	84.00	2.85		CTX
IA22	<0.1	n.p.	0.16	0.45	<0.1	<0.1	542.00	10.60		Negative
IA23	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	122.00	2.36	Pork meat pos.	CTX/IFX
IA24	0.83	n.p.	<0.1	0.13	n.p.	n.p.	79.50	n.p.		CTX
IA25	<0.1	n.p.	<0.1	<0.1	<0.1	<0.1	2.18	9.59		Negative
IA26	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	53.70	20.90	Pork meat pos.	Negative
IA27	<0.1	n.p.	<0.1	<0.1	<0.1	<0.1	22.00	1.10		Negative

ImmunoCAP®										
ID	CTX	Bos d TG	Pork	Beef	Pig urine proteins	Bos d 6	Total IgE	Tryptase (µg/L)	Provocation	IgE pattern (dot blot)
IA28	21.5	16.5	0.56	1.00	0.91	<0.1	138.00	5.20	Innards/meat pos.	CTX/HSA-α-Gal2 + 3/Bos d TG
IA29	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	175.00	6.20		Negative
IA30	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	10.20	3.40		TG
IA31	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	1.10	4.00		Negative
IA32	0.68	<0.1	<0.1	<0.1	<0.1	<0.1	75.00	5.40		CTX
IA33	4.98	4.44	<0.1	<0.1	<0.1	<0.1	140.00	2.80		CTX/HSA-α-Gal3/Bos d TG
IA34	n.p.	<0.1	<0.1	<0.1	<0.1	<0.1	1670.00	4.40		CTX/IFX/Bos d TG

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 IgE (kU/L)

ImmunoCAP®											
ID	CTX	Bos d TG	IFX	Pork	Beef	Bos d 6	Pig urine proteins	Total IgE	Tryptase (µg/L)	Allergy status	IgE pattern (dot blot)
HC1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	2.8	4.0	–	Negative
HC2	<0.1	<0.1	n.p.	<0.1	<0.1	<0.1	<0.1	6.2	13.2	–	Negative
HC3	0.14	<0.1	n.p.	<0.1	<0.1	<0.1	<0.1	199	3.4	A	Negative
HC4	n.p.	<0.1	n.p.	<0.1	<0.1	<0.1	<0.1	35.3	4.7	–	Bos d TG
HC5	n.p.	<0.1	n.p.	<0.1	<0.1	<0.1	<0.1	5.0	2.9	–	Negative
HC6	n.p.	<0.1	n.p.	<0.1	<0.1	<0.1	<0.1	9.3	5.4	–	Negative
HC7	n.p.	<0.1	n.p.	<0.1	<0.1	<0.1	<0.1	27.3	3.1	–	Bos d TG
HC8	n.p.	<0.1	n.p.	<0.1	<0.1	<0.1	<0.1	6.3	5.9	–	Negative
HC9	<0.1	<0.1	n.p.	<0.1	<0.1	<0.1	<0.1	58.5	3.3	A	Negative
HC10	n.p.	<0.1	n.p.	<0.1	<0.1	<0.1	<0.1	115	5.7	A	Negative
HC11	0.11	0.18	n.p.	<0.1	<0.1	<0.1	<0.1	334	3.9	A	Negative
HC12	n.p.	<0.1	n.p.	<0.1	<0.1	<0.1	<0.1	66.8	5.9	–	Negative
HC13	n.p.	<0.1	n.p.	<0.1	<0.1	<0.1	<0.1	8.1	5.4	–	Negative
HC14	n.p.	<0.1	n.p.	<0.1	<0.1	<0.1	<0.1	80.1	5.2	–	Negative
HC15	<0.1	<0.1	n.p.	<0.1	<0.1	<0.1	<0.1	<2.0	1.9	–	Bos d TG
HC16	n.p.	<0.1	n.p.	<0.1	<0.1	<0.1	<0.1	70.3	4.1	A	Bos d TG
HC17	n.p.	<0.1	n.p.	<0.1	<0.1	<0.1	<0.1	10.5	4.3	–	Bos d TG
HC18	n.p.	<0.1	n.p.	<0.1	<0.1	<0.1	<0.1	10.3	4.2	A	Negative
HC19	n.p.	<0.1	n.p.	<0.1	<0.1	<0.1	<0.1	175	4.2	A	Negative
HC20	n.p.	<0.1	n.p.	<0.1	<0.1	<0.1	<0.1	49.3	5.4	–	Negative
HC21	<0.1	<0.1	n.p.	<0.1	<0.1	<0.1	<0.1	20.3	1.9	–	Negative
HC22	n.p.	<0.1	n.p.	<0.1	<0.1	<0.1	<0.1	52.4	9.4	A	Negative
HC23	n.p.	<0.1	n.p.	<0.1	<0.1	<0.1	<0.1	423	3.0	A	Negative
HC24	0.20	<0.1	n.p.	<0.1	<0.1	<0.1	<0.1	58.8	6.9	–	Negative
HC25	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	476.7	3.3	–	CTX/IFX
HC26	n.p.	<0.1	n.p.	<0.1	<0.1	<0.1	<0.1	39.2	3.8	–	Negative

ImmunoCAP®											
ID	CTX	Bos d TG	IFX	Pork	Beef	Bos d 6	Pig urine proteins	Total IgE	Tryptase (µg/L)	Allergy status	IgE pattern (dot blot)
HC27	n.p.	<0.1	n.p.	<0.1	<0.1	<0.1	<0.1	105	8.1	–	Negative
HC28	n.p.	<0.1	n.p.	<0.1	<0.1	<0.1	<0.1	26.7	3.1	–	Bos d TG
HC29	n.p.	<0.1	n.p.	<0.1	<0.1	<0.1	<0.1	87.3	6.4	–	Negative
HC30	n.p.	<0.1	n.p.	<0.1	<0.1	<0.1	<0.1	17.4	8.6	–	Bos d TG
HC31	<0.1	<0.1	n.p.	<0.1	<0.1	<0.1	<0.1	41.1	3.9	–	Negative
HC32	17.3	16.1	0.14	1.58	3.01	<0.1	2.95	39.04	4.3	–	CTX/HSA-α-Gal2 + 3/Bos
HC33	n.p.	<0.1	n.p.	<0.1	<0.1	<0.1	<0.1	46.7	13.2	A	Negative
HC34	<0.1	<0.1	n.p.	<0.1	<0.1	<0.1	<0.1	8.4	6.3	–	Negative
HC35	0.11	<0.1	n.p.	<0.1	<0.1	<0.1	<0.1	188	7.6	A	Negative
HC36	n.p.	<0.1	n.p.	<0.1	<0.1	<0.1	<0.1	106	1.9	–	Negative
HC37	<0.1	<0.1	n.p.	<0.1	<0.1	<0.1	<0.1	4.1	6.5	–	Negative
HC38	<0.1	<0.1	n.p.	<0.1	<0.1	<0.1	<0.1	7.4	5.0	A	Negative
HC39	<0.1	<0.1	n.p.	<0.1	<0.1	<0.1	<0.1	102	4.4	A	Bos d TG
HC40	4.46	1.73	<0.1	<0.1	0.39	<0.1	<0.1	165	3.6	–	CTX