UNIVERSITÉ DU LUXEMBOURG

PhD-FSTM-2023-09
The Faculty of Science, Technology and Medicine

DISSERTATION

Defence held on 02/02/2023 in Esch-sur-Alzette, Luxembourg

to obtain the degree of

DOCTEUR DE L'UNIVERSITÉ DU LUXEMBOURG

EN BIOLOGIE

by

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MOLECULAR FACTORS INVOLVED IN TICK-BITE MEDIATED ALLERGY TO THE CARBOHYDRATE α-GAL

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AFFIDAVIT

I hereby confirm that the PhD thesis entitled “Molecular factors involved in tick-bite mediated allergy to the carbohydrate α-Gal” has been written independently and without any other sources than cited.

Luxembourg, 30.11.2022

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ACKNOWLEDGEMENTS

First and foremost, I would like to thank Dr. Christiane Hilger for her supervision and for critical review of the work. Most of all I thank you for giving me space to do things at my own pace and for the freedom to explore my own ideas. I have truly learnt to be an independent researcher under your guidance. I would also like to thank Dr. Iris Behrmann and Dr. Simon Blank – my CET committee members, for your valuable suggestions and for your time.

Thank you Dr. Markus Ollert and Dr. Annette Kühn for your valuable feedbacks over the years, which have certainly helped me improve my work. Thanks to all the collaborators of the 'Project IRGal' for insightful discussions and for four years of working together. Lastly, but very importantly thanks to Dr. Gunnar Dittmar for help with the proteomics analysis.

Thanks to my team ALL2 for the four years of working together, for your help, and for the companionship. Thank you Kyra Swiontek for your help and for contributions to the work done in the frame of this thesis. Special thanks to Tanja Scheuermann for always being so kind, it really meant a lot. Thank you Dominique Revets for all the funny and interesting discussions.

Thanks to all my friends who have been there, unfortunately, most of you were far in distance but that doesn't in anyway diminish the role you have played during these four years. Special thanks to Vikas, for your advices and for inspiring me so much simply by the virtue of who you are.

Lastly, but most importantly my deepest gratitude to my family for their love and support throughout and for always taking pride in what I do. Special thanks to Anurag, my brother, for the love you have and to Robin for who you are and for filling my days with laughter.

Thank you Mooji and Papaji.

For my parents…

Smt. Asha and Shri Yaduvansh Yadav

Nature does not hurry, yet everything is accomplished

- Lao Tzu

SCIENTIFIC CONTRIBUTIONS

Published

"α-Gal present on both glycolipids and glycoproteins contributes to the immune response in meat-allergic patients" – *The Journal of Allergy and Clinical Immunology; Volume 150, Issue 2, P396-405.E11, August 01, 2022.*

"Neera Chakrapani, Jörg Fischer, Kyra Swiontek, Françoise Codreanu-Morel, Farah Hannachi, Martine Morisset, Clément Mugemana, Dmitry Bulaev, Simon Blank, Carsten Bindslev-Jensen, Tilo Biedermann, Markus Ollert, Christiane Hilger"

DOI: https://doi.org/10.1016/j.jaci.2022.02.030

In preparation

I. Anti α-Gal antibody responses in a cohort of Luxembourgish forestry employees show distinct IgG and IgG subclass profiles – *manuscript in preparation*

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II. Mouse blood protein survives degradation in *Ixodes ricinus* ticks after moulting and upon starvation up to five months – *manuscript in preparation*

Chakrapani et al.

Co-author

"A novel monoclonal IgG1 antibody specific for Galactose-alpha-1,3-galactose questions alpha-Gal epitope expression by bacteria" – *Frontiers in Immunology; Sec. Microbial Immunology, August 05, 2022.*

"Luisa Kreft, Aloys Schepers, Miriam Hils, Kyra Swiontek, Andrew Flateley, Robert Janowski, Mohammadali Khan Mirzaei, Michael Dittmar, **Neera Chakrapani**, Mahesh S. Desai, Stefanie Eyerich, Li Deng, Dierk Niessing, Konrad Fischer, Regina Feederle, Carsten B. Schmidt-Weber, Simon Blank, Christiane Hilger, Tilo Biedermann, Caspar Ohnmacht"

DOI: https://doi.org/10.3389/fimmu.2022.958952

INDEX

ABBREVIATIONS

α-Gal	Galactose-α-1,3-galactose
α-Gal HSA	α-Gal conjugated to human serum albumin
AP-N	Aminopeptidase N
AUC	Area under the curve
BAT	Basophil activation test
BL	Beef lipids
CD	Cluster of differentiation
CL	Chicken lipids
EC50	Half maximal effective concentration
ELISA	Enzyme-linked immunosorbent assay
FA	Food allergy
FcεRI	High affinity IgE receptor
GI	Gastro-intestinal tract
GL	Glycolipid
GP	Glycoprotein
IC50	Half maximal inhibitory concentration
IgE, IgG, IgM	Immunoglobulin isotypes E, G, M
IL	Interleukin
LC-MS	Liquid chromatography-mass spectrometry
Mab	Monoclonal antibody
MALDI-TOF	Matrix-assisted laser desorption/ionization – time of flight
M86	Anti-α-Gal IgM
PK	Pork kidney
sIgE	Specific IgE
TLC	Thin layer chromatography

SUMMARY

Prevalence of food allergy is sharply rising in the western world and contributes to an increase in the healthcare burden. It also puts affected individuals at risk and strongly impacts their quality of life. In the past decade, red meat allergy has emerged as an interesting phenomenon in food allergy due to several distinguishing features. Most importantly, a delayed allergic reaction occurring 2-6 hours after food consumption, secondly, involvement of a carbohydrate allergen (galactose-α-1,3-galactose), thirdly, sensitization via tick bite, making it the first vector borne allergy to be recognized. Another important aspect is the involvement of the skin-gut axis in the route of sensitization and allergic response. Although humans have a natural IgG response to α-Gal that is thought to arise from antigenic stimulation by gut microbiota, tick bites, most probably due to adjuvant molecules present in saliva, are able to break tolerance to α-Gal and trigger red meat allergy. In general, food allergies are the result of an immune response directed towards a protein allergen, and they elicit an immediate onset of allergic symptoms, usually within 30 minutes. These enigmatic features of red meat allergy warrant further investigation into molecular and immune mechanisms giving rise to the associated features.

The work done in this thesis aims at elucidating factors involved in tick bite sensitization and investigating the molecules involved in the delayed allergic response upon ingestion of mammalian meat. In **Chapter I,** I have explored the sensitization prevalence to α-Gal in a high-risk population of Luxembourgish forestry employees. Further, we have analysed total IgE levels and variation in subclasses of specific IgG to α-Gal and compared the antibody profile to allergic patients. We observed a distinct IgG response against α-Gal in sensitized employees and meat-allergic patients compared to the control group, supporting a direct role of tick bites in boosting specific IgG1 and IgG2 responses. Secondly, I have looked into the source of sensitizing molecules in *Ixodes ricinus* – a major causative tick in Europe. In **Chapter II**, we have used proteomics analysis to detect mouse blood proteins in a longitudinal study by feeding ticks on BALB/c mice and detecting mouse protein after moulting and starvation. We detected large quantities of mouse protein even after prolonged starvation of five months. Thereby, showing that residual host protein could be a source of sensitizing molecules.

Lastly, in **Chapter III** I have explored in depth the allergenic potential of α-Gal on glycolipids. Several other related questions such as stability of glycoproteins and mechanistic behind delayed onset of symptoms were investigated using a model glycoprotein as well as extractions from natural food sources. We found glycolipids to be allergenic *in vitro*, they bound IgE and activated basophils from meat-allergic patients. Glycoproteins were shown to be highly stable and detectable for up to 4 hours in simulated gastrointestinal digestion. The slow breakdown into smaller glycopeptides, a prerequisite for intestinal absorption, is very much in line with the observed delayed allergic response.

Through the work presented here, I have managed to shed light onto three important aspects of α-Gal allergy – a direct role of tick bites in boosting specific IgG responses to α-Gal and a higher sensitization prevalence in a high-risk population. Secondly, in view of the debate on the source of sensitizing molecules in ticks we show the presence of large quantities of host-derived proteins for prolonged periods, thereby, supporting a non-human blood meal based origin of the sensitizing allergen. Lastly, the detailed characterization of glycolipids and glycoproteins from mammalian food illustrates the crucial role of molecule stability and abundance in the delayed response and severity of symptoms.

INTRODUCTION

I. Food Allergy

Food allergy (FA) refers to a pathological immune response in allergic individuals against an otherwise harmless antigen present in the food consumed. Patients suffering from FA develop an aggravated immune response to harmless food antigen, which can result in an immediate reaction, occurring within minutes to up to 2 hours after ingestion of the implicated food [1]. There has been a sharp rise in the incidences of FA over the past few decades and it accounts for approximately 9% of all allergies reported today. Interestingly, more than 90% of food allergies are associated with a handful of allergens famously termed as 'the Big-8' namely – soy, peanut, milk, eggs, fish, tree nuts, wheat, and crustacean shellfish [2]. Symptoms in allergic individuals can range from milder ones like pruritus, swelling in throat and mouth, diarrhea, hives etc. to a more severe form of life-threatening anaphylactic shock. FA not only represent a major economic and healthcare burden but also affect an individual's quality of life. Daily management of FA can be a challenging task for patients, not only reducing their food choices but also serving as an added stress factor affecting their day-to-day life choices.

1.1. Epidemiology of food allergy

Global prevalence of FA ranges from 1.1% to 10.8% [3]. Most cases are reported from Europe, US, UK, and Australia, and number of cases from other parts of the world are increasing with the incorporation of a westernized lifestyle [4]. As per 'European Academy of Allergy and Clinical Immunology' (EAACI) manifesto, currently 150 million Europeans suffer from chronic allergy and this number will increase to affect 50% of the European population by 2025 [5]. Based on data from the EuroPrevall (Prevalence, cost and basis of food allergy in Europe) study 1.9% to 5.6% of children and 0.3% to 5.6% of adults in Europe are reported to suffer from probable FA [6]. In US, a population based survey reports a prevalence rate of 8% and 11% in children and adults respectively [7], whereas Australian studies such as HealthNuts and SchoolNuts report prevalence rates as high as 10% among infants and 4% to 5% in children and youths [4]. Milk and egg allergies are most common among children and usually outgrown with age, whereas peanut and tree nut allergies tend to persist [8]. Adults more often report allergies to fruits and vegetables (oral allergy syndrome), peanuts and tree nuts, and fish and shellfish [9, 10]; however, it is likely that most of them have had the said allergy since childhood. An important exception here is the oral food syndrome that can develop in adulthood and is often a result of cross-reactivity between allergens in pollen, raw fruits, vegetables and sometimes tree nuts [10]. The associated symptoms are usually short lived and can be helpful in distinguishing a secondary food allergy from an actual primary FA.

1.2. Clinical manifestations

Onset of allergic reactions occur within minutes to up to 2 hours after food consumption, and most common symptoms include disturbances in the gastrointestinal tract (GI), skin, respiratory tract and cardiovascular abnormalities. GI related symptoms comprise of responses such as abdominal pain, diarrhea, nausea and vomiting; skin reactions including urticaria (hives), angioedema and erythema and respiratory symptoms including bronchospasm, laryngeal edema etc. are the common clinical manifestations [11-13]. Anaphylaxis is the most severe manifestation of FA that is rapid and can lead to death in the absence of immediate medical attention. It is of note, cow's milk, peanut, eggs, fish, tree nuts and shellfish are the most frequently reported foods causing anaphylaxis, however any food can lead to anaphylactic shock [12].

Based upon immune mechanism involved FA can be categorized into IgE-mediated, non-IgE mediated and mixed (IgE/non-IgE-mediated) responses. Non-IgE mediated reactions such as food protein-induced enterocolitis syndrome (FPIES), eosinophilic esophagitis, or food protein-induced proctocolitis are mediated by the innate immune response and T lymphocytes, and primarily affect the GI whereas IgE-mediated reactions are driven by immunoglobulin E (IgE) bound to the surface of mast cells and basophils, resulting in local as well as systemic reactions [11, 13]. IgE-mediated allergic responses are discussed further; non-IgE-mediated reactions are beyond the scope of this thesis and hence not discussed.

1.3. Mechanisms

Food allergies are a result of the breakdown of oral tolerance to an otherwise harmless ingested food. Oral tolerance is the development of a tolerogenic immune response to an antigen upon its first encounter in the gastrointestinal (GI) tract [14].

Oral tolerance: Development of oral tolerance in the gastrointestinal tract depends on several characteristics such as the digestive processes, intact epithelial barrier, immune modulation and presence of specific immune cells. Normal response to food antigen leads to development of immune tolerance towards the ingested food due to the involvement of specialized cells like microfold, intestinal epithelium cells and dendritic cells present in the GI tract [15, 16]. Dendritic cells play a major role in tolerance induction and maintenance, by migrating to mesenteric lymph nodes upon antigen uptake and promoting generation of Treg cells specific to the food antigen. Tregs lead to a tolerant immunological response by producing TGF-β and IL10. TGF-β suppresses T and B-cell activity whereas IL10 promotes T-cell anergy and maintains Treg population [17].

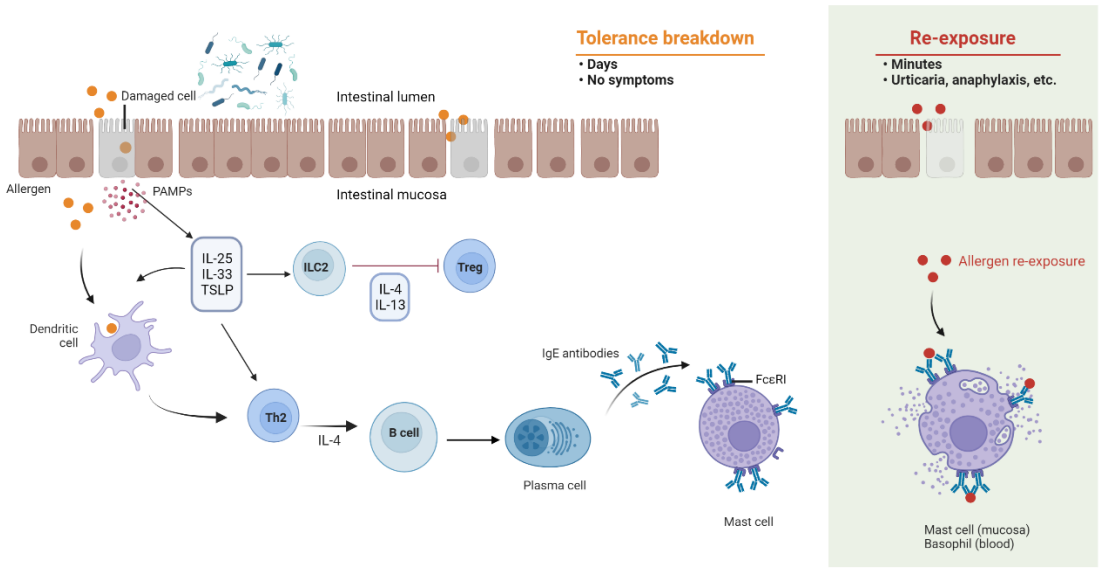

Figure 1. Mechanisms of food allergy – Tolerance breakdown: tolerance is broken upon encounter of danger signals such as pathogen-associated molecular patterns (PAMPs) or in response to epithelial cell damage which can result in expression of IL-25, IL-33, and thymic stromal lymphopoietin (TSLP). Subsequently, resulting in dendritic cells differentiation into a Th2-favouring phenotype; in turn, Th2 cells produce IL4 that simulates various aspects of the allergic response, importantly class-switching of B cells into IgE producing plasma cells. Additionally, innate lymphoid cells (ILC2) block Treg cell function by secreting IL-4 and IL-13. IgE secreted by plasma cells binds to the mast cells and basophils. In a secondary allergen exposure (right), allergen-mediated cross-linking of cell-surface bound IgE can occur, thereby resulting in an onset of allergic reactions [11]. Created in Biorender.

Sensitization: Sensitization to a food allergen takes place during the first encounter with the allergen via the gastrointestinal tract or skin (in some cases), sometimes it can occur through the respiratory tract upon encountering an impaired/inflamed barrier [11, 14, 18-20]. A damaged epithelial barrier can lead to disruption in tolerance due to increased exposure to factors like pathogen-associated molecular patterns (PAMPs), moreover, inflammation or epithelial damage in gut (sometimes, skin or airways as well) can lead to increased penetration of antigen thereby promoting the release of cytokines IL25, IL33, and TSLP driven by the epithelium. This consequently skews the immune system towards a Th2 (T helper 2 cells) response and promotes differentiation of dendritic cells (DC) into a Th2-favouring phenotype which further orchestrates the differentiation of naïve $CD4^+$ T-cells. Th2 cells produce IL4, a key cytokine that sustains Th2 response and drives the B-cell switching to the production of IgE. In addition, Tuft cells secrete IL25 that can aid in the proliferation of ILC2 (innate lymphoid cells) populations, that further contributes to the pro-allergic response by secreting cytokines IL4 and IL13 thereby blocking the functioning of Treg cells [11].

Food antigen-specific IgE binds to the high-affinity Fc receptor (FcεRI) on mast cells and basophils. Subsequent encounter with the food allergen leads to IgE cross-linking upon allergen binding and results in activation of the mast cells in tissues and basophils in blood circulation [21]. The activated mast cells and basophils release IL4 and IL13 promoting survival of Th2 cells and suppression of Tregs [22]. In addition, release of preformed mediators such as histamine, leukotrienes, prostaglandins, tryptase etc. result in hypersensitivity reactions. In the GI tract, this could manifest in the form of responses such as increased contraction, mucosal secretion, diarrhea, vomiting etc. Whereas systemic distribution of released mediators via bloodstream to far away tissues can result in varied symptoms in different organs of the body as described previously [7, 11, 14, 17, 21].

Dual allergen-exposure hypothesis

Dual allergen-exposure refers to a phenomenon where different routes of exposure are involved in allergic sensitization and elicitation of the response. Allergic response is usually reported to involve the same sensitization and elicitation route. However, early observations in infants with eczema led to the formulation of dual allergen-exposure hypothesis, when the infants were found to develop sensitization to food antigen through environmental exposure via disrupted skin barrier [23]. High prevalence rates of peanut allergy observed in infants with atopic eczema is a widely reported case of dual allergen-exposure; follow up studies in allergic infants showed induction of tolerance upon early introduction of peanuts (LEAP study) [24]. It is now well established that sensitization to food can occur through routes other than oral [11]. Other allergies identified so far that conform to the concept of dual-allergen hypothesis include – α-Gal allergy which is a delayed allergic response to red meat developed upon sensitization by tick bites and result in hypersensitivity reactions upon encounter of α-Gal in the mammalian meat by oral route. Another example of interest is allergy to donkey's milk, wherein patients get sensitized through skin upon using cosmetics containing donkey's milk as an ingredient and as a consequence develop allergic reaction upon consuming donkey's milk [25].

1.4. Antibodies in allergic response

Immunoglobulin E

IgE is the least abundant immunoglobulin, constituting approximately 0.05% of the total immunoglobulin in serum and has the shortest half-life (2-3 days). It is composed of a heavy chain (ε) with four constant domains Cε1-Cε4 and 1 variable region [26]. It serves as a first line of defence in protection against toxins and endoparasites, and thus plays a pivotal role in protection from parasitic infections such as helminth infections [27]. Elevated titers of IgE is a hallmark of type I hypersensitivity reaction in atopic individuals upon development of an allergic disease. IgE binds to the FcεR receptor on cell-surface, which are of two types – high-affinity receptors (FcεRI), and low-affinity receptors (FcεRII). FcεRI is expressed on mast cell, basophils, eosinophils, and langerhans cells and FcεRII (CD23) is expressed on the same cells as FcεRI and additionally on B cells, dendritic cells, monocytes, macrophages, and platelets [21].

When exposed to a harmless environmental allergen, susceptible individuals can mount an IgE-driven immune response against the allergen. Serum of an allergic individual contains allergen-specific IgE (sIgE), which indicates a prior exposure to allergens. These IgE antibodies bind to the FcεRI receptor on mast cells and basophils on exposure to allergens, resulting in cross-linking of surface bound IgE, mediators are released causing an onset of allergic response. For example, mast cell mediators released by an activated mast cell rapidly increase smooth muscle contraction and vascular permeability, which are common symptoms of an allergic reaction [26].

Immunoglobulin G

IgG is the most abundant antibody isotype and constitutes 70% to 75% of the serum immunoglobulin. It is comprised of four different subclasses – IgG1 (66%), IgG2 (23%), IgG3 (7%), and IgG4 (4%), that exhibit distinct effector function due to different heavy chains [28]. IgG1 and IgG3 activate complement and are responsible for clearance of most of pathogens; while IgG2 can activate complement only in presence of high quantities of antigen; IgG4 isn't involved in complement activation at all. IgG2 is chiefly produced against thymus-independent antigens, and IgG4 along with IgE are normally produced either upon allergen exposure or during helminth infection [29]. Upon encounter of an antigen healthy individuals can produce specific-IgG. Significance of IgG in allergy is an emerging concept, it has been explored in studies on respiratory, food, and venom allergens in immunotherapy. These studies have shown modulation in basophils and mast cell activity due to the blocking activity of IgG antibodies [30]. Study in an *in vitro* model of grass pollen allergy showed reduction in basophil activation due to the presence of blocking antibodies of different isotypes but with similar specificity as IgE [31]. Recently, McKendry et al. analyzed role of inhibitory mechanism of IgG in the context of FA and found different patterns of allergen-specific IgG subclasses in children with persistent *versus* transient FA; they attribute this reduction in allergic symptoms to the blocking activity of the IgG antibodies [32]. However, more work needs to be done to clearly establish role of specific-IgG in inducing tolerance in allergic patients.

Role of IgG4 in allergy: Serum IgE levels are often used to predict severity of allergic reaction, however many individuals with elevated sIgE don't develop allergic response upon oral food challenge. In addition, sensitized individuals are tolerant to implicated food/allergen, indicative of the role of specific antibodies of other isotypes in reduced/absent reactivity of basophils and mast cells [33]. The most explored and important candidate in inducing tolerance is IgG4 due to its distinct structural properties and non-complement activating feature. It plays a major role in natural resolution of allergy

and is related to a positive outcome in immunotherapy. Much like IgE, production of IgG4 is induced in presence of IL-4 wherein the protective mechanism of IgG4 is attributed to its ability to exist in a monovalent form. It binds to free allergen in blood and facilitate allergen clearance, thereby reducing available antigen for IgE binding on mast cells and basophils. In addition, half antibodies from different IgG4 molecules can join via fab arm exchange (FAE) mechanism resulting in bi-specific antibodies, thus allowing recognition of two epitopes on an allergen molecule that can further increase cross-linking of allergen molecules and clearance [34]. Ratios such as IgG/IgE and IgG4/IgE are frequently used in clinical settings to gain important insight into progression/resolution of allergy [33].

1.5. What makes a food allergen allergenic?

Antigenic potential of an allergen molecule determines its role in sensitization and induction of an allergic response upon secondary exposure. Antigenicity depends upon 2 factors – immunogenicity and allergenicity. An antigen is considered an immunogen when it has a potential to activate the immune system to either attack, develop tolerance, or maintain homeostasis. A normal immunogenic response is Th1 driven and results in a pro-inflammatory response, whereas allergenicity refers to the capacity of a molecule to initiate a Th2 response towards a harmless molecule. Since an allergenic molecule is usually tolerated by the immune system, individuals developing a response towards them are often predisposed to do so. This predisposal can depend upon myriad factors such as genetic, environmental, early-life exposure etc. [35]

Allergenicity of a molecule determines its IgE-binding potential and severity of clinical symptoms. The allergenicity of an antigen (food) in return is dictated by its physicochemical properties, which plays a deciding role by altering the biochemistry of the molecule. Physicochemical properties such as ability to survive extreme processing environment, resist digestion in the gastrointestinal tract and retain integral epitope long enough to interact with the immune system, are important contributing features [36, 37]. All most all of the food allergens identified to date are proteins and common features contributing to increase their allergenic potential are –

Abundance – Allergen abundance increases its chances of encounter with the immune system, whereas most low abundant food allergen might not be able to survive proteolytic digestion, allergens present in large quantities can survive and reach blood stream to trigger a response. Major allergens from common allergenic food such as milk, eggs, soy, nuts etc. constitute >1% of the food protein [38]. Major shrimp allergen, Pen a 1, accounts for 20% of the total shrimp-tail protein [39], whereas Ara h 1 and Ara h 2, the major peanut allergens account for 2% and 6% of the total protein respectively [38]. Several other factors (e.g. stability) can increase survival of allergen thereby making them stay for a longer time and induce a reaction. However, abundance is a predisposing factor which when combined with additional features can make a food protein allergenic [36].

Allergen structure and post-translational modifications – Allergen structure is often considered an important factor in increasing its resistance to denaturation; wide occurrence of di-sulfide bonds in food allergens [40] further strengthens this hypothesis. Disruption of di-sulfide bonds on β-lactoglobulin (milk allergen) decreased the allergen stability [41]. Additionally, structural changes in Ara h 1 and Ara h 2 (peanut allergen) were found to alter the allergenicity of the allergen molecules by making them more susceptible to proteolysis [42, 43]. Secondly, glycosylation is a common modification (post-translational) among animal allergens such as caseins, serpins, ovomucoids, etc.

The vicilin family contains the major glycosylated plant allergens reported so far [36]. Oligosaccharides covalently attached to the asparagine or serine/threonine residues commonly termed as N-linked and O-linked glycosylation respectively are widely present on many allergens, and they can increase the immunogenicity and allergenicity of a protein allergen by altering its physicochemical properties such as solubility, stability, electrical charge etc. [44]. Although in most cases removal of glycosylation decreases the allergenicity, in some cases an increase in the allergenic potential has been observed; of note, there are many allergens where the role of glycosylation is not yet clear [36].

Resistance to digestion – Resistance to digestion and denaturation is a key feature of food allergens that enables them to survive food processing and gastrointestinal digestion. *In vitro* digestion is therefore a preferred method to study allergen stability and availability to the system. Many food allergens tested so far, were found to be resistant to enzymatic digestion [45]. In an interesting exception, a lack of stability has been observed among cross-reactive allergens from fruits such as Pru av 1 (cherry), Mal d 1 (apple) etc., which can cause symptoms localized to the throat e.g. swelling of lips, tongue, or glottis but no gastro-intestinal manifestation as observed in most food allergies [46].

Epitope configuration – IgE cross-linking is a pre-requisite for allergic response and due to this requirement, an allergen molecule should have at least 2 epitopes in order to facilitate IgE cross-linking which then leads to mediator release by the activated cells and a subsequent onset of allergic reactions.

In a protein allergen, epitopes can be present in two forms –

i. linear – primary amino-acid sequence binds to the IgE
ii. conformational – secondary or tertiary protein structures where a unique combination of amino acids come together to form the epitope

In FA, linear epitopes, normally peptides with 6 to 15 amino acids play a major role due to higher stability in the gastrointestinal digestion. Conformational epitopes might not be very stable whereas linear epitopes become available for immune recognition after partial digestion. Linear epitopes have been mapped for multiple allergens now, including most common allergen sources – milk, soy, peanuts etc. and are highly interesting in *in vitro* prediction of the severity of allergic reactions [36, 37, 47].

Ligand binding – Recent findings show a relevant role of small molecules such as lipids, steroids, plant hormones etc., and allergen ligands in the initiation of an allergic response by altering allergen properties such as protein stability, influencing IgE binding activity, oligomerization etc., thus contributing to the onset of allergic sensitization and progression of the effector phase. These molecules can occur in the matrix surrounding the allergen, such as food matrix, pollen matrix, and animal/plant derived dust. Additionally, they can interact directly or co-localize with the allergen molecule and affect its reactivity [48]. The interaction of a ligand with an allergen molecule can lead to conformational changes and therefore affect its stability against thermal denaturation, lysosomal degradation, and gastric stability etc., thereby prolonging allergen availability for interaction with the immune cells. Moreover, lipids in conjugation with allergens can alter inflammatory responses by acting as a promotor/enhancer and leading to activation of certain T-cell subsets by CD1 presentation thereby contributing to the allergic sensitization [49, 50].

1.6. Red meat allergy

Red meat is widely consumed all across the world and beef, pork, lamb, ham, goat etc. are the most commonly consumed form of meat. Although, a wide variation in choice of meat could be seen from region to region, for example consumption of innards is popular in Europe whereas it is less common in other parts of the world. In addition to meat, several processed forms of meat such as bacon, salami, sausages etc. and derived products like liver paté, fat, and gelatin are widely used. Allergy to red meat is considered a rare form of allergy, cases have been reported mostly in children and majority of them are often sensitized to the bovine serum albumin. Reports of red meat allergy among adults remained low until the discovery of α-Gal allergy, which has emerged as a major form of red meat allergy.

Red meat allergens: despite widespread consumption of meat from animals such as goat, sheep, and deer, no major allergen has been identified. In case of rabbit and guinea pig, around four or five allergens have been reported in the IUIS database, more importantly these allergens were identified based on inhalant symptoms. So far, cow remains the only species with significant number of identified food allergens (ten allergens), majority of the allergens were identified in cow's milk [50]. Allergy related to many meat products such as goat and sheep are less commonly reported, which could also be attributed to under diagnosis as meat from certain animals are preferentially consumed in different regions across the world. Three major forms of red meat allergy recognized so far are – beef allergy, pork-cat syndrome and α-Gal allergy. Beef allergy and pork-cat syndrome are a result of cross-reactivity, whereas α-Gal allergy is a primary meat allergy and patients sensitized to α-Gal react to the carbohydrate allergen galactose-α-1,3-galactose when encountered in the consumed mammalian products.

In beef allergy, Bos d 6 (bovine serum albumin) and Bos d 7 (bovine immunoglobulin, actin, myoglobin) are the major allergens that have been reported; due to the wide occurrence of Bos d 6 in muscle meat as well as milk, many patients with beef allergy report allergic responses to milk as well [51, 52]. The allergic reaction often occurs rapidly with onset of symptoms such as urticarial, vomiting, nausea etc. However, most children develop tolerance with age and eventually outgrow allergy [53]. Second interesting type of meat allergy is pork-cat syndrome, in this case cat-allergic patients develop a reaction to consumed pork meat due to cross-reactivity between the sensitizing allergen Fel d 2 (cat serum albumin) and the ingested allergen Sus s 1 (pork serum albumin) [54]. Additionally, IgE from some patients have been reported to bind to Bos d 6 (bovine serum albumin) causing reactions to beef and horse meat as well [50]. Pork-cat syndrome is predominantly reported in adolescents and young adults and the reaction occurs within an hour of pork ingestion thereby setting it apart from more delayed form of red meat allergy i.e. α-Gal allergy. For detailed description on α-Gal allergy, please see next chapter.

II. The α-Gal allergy – a novel food allergy

The α-Gal allergy is a newly recognized carbohydrate-mediated allergic reaction that results in a delayed form of allergic response upon antigen encounter via oral route, and an immediate onset of hypersensitivity reactions has been observed upon subcutaneous administration of mammal-derived biologicals/colloids etc. Patients develop specific IgE directed against the carbohydrate epitope galactose-α-1,3-galactose (α-Gal) upon sensitization via tick bite. Allergic onset is commonly observed in adults, they report sudden development of allergic response usually after eating red meat without any complication for several years. Hard ticks have been found to be a causative agent, however exact sensitization mechanism is not fully understood [55-61].

2.1. Non-IgE responses to α-Gal

Carbohydrate galactose-α-1,3-galactose (α-Gal), is present in all mammals except humans, apes, and old-world monkeys due to the loss of α-1,3-galactosyltransferase (α-1,3-GT) activity owing to inactivation of the GGTA1 gene by a frameshift mutation [62]. This mutation is thought to have provided an evolutionary advantage by conferring protection against pathogens carrying α-Gal or α-Gal-like structures, as absence of endogenous α-Gal allows production of anti-α-Gal IgG, IgM, and IgA antibodies in humans [63-67]. These antibodies play a protective role in infection against bacteria and pathogens by recognizing α-Gal present on their surface and preventing immune invasion by complement activation. Enveloped viruses expressed in engineered human and animal cells with active α-1,3-GT presented numerous surface bound α-Gal epitopes, and were more effectively lysed by the human anti-Gal antibodies and complement [68, 69]. α-Gal epitopes have been identified on parasites such as *Leishmania braziliensis*, *Trypanosoma cruzi*, and *L. Mexicana* [63]; patients suffering from leishmaniasis and Chagas' disease have been reported to have elevated levels of anti-α-Gal antibodies [63], pointing towards a possible immunogenic stimulation by these parasites. Additionally, elevated anti-Gal IgM, IgG, and IgA have been reported in patients with Crohn's disease [70] and lower levels of anti-Gal IgM and IgG has been reported in patients with Guillain-Barré syndrome and Alzheimer's disease compared to healthy individuals (Pacheco et al., 2021; Angiolillo et al., 2021). Hence, clearly demonstrating immunological significance of the anti-α-Gal antibodies in humans.

In an early study, Hamanova et al. identified anti-α-Gal IgM, IgG, and IgA antibodies in human subjects and found high quantities of IgA in secretions [71]. The anti-α-Gal IgA in secretions is shown to play a protective role by binding *Neisseria meningitidis* [72]. Moreover, higher titers of anti-α-Gal IgM in malaria-endemic region correlated with decreased susceptibility to malaria infection [73] and high levels of anti-α-Gal IgG/IgM were reported in healthy individuals compared to those infected with *Mycobacterium tuberculosis* or *Plasmodium falciparum* [73]. Thus clearly showing a protective role of anti-α-Gal antibodies in pathogenic infections. Early studies conducted to determine titer of anti-Gal antibodies reported as much as 1% of the circulating antibodies directed against α-Gal [67, 74]; however, some studies have now found the quantities to be less, roughly 0.1-0.2% of the immunoglobulins, than previously reported [75-77].

Anti-α-Gal IgM/IgG antibodies owing to higher abundance in humans are an emerging immunological tool with several applications in treatment and preventive measures, most relevant one being its role in treatment of cancer [78, 79]. The anti-α-Gal antibody production is proposed to be induced by commensal bacteria in the gut as an immunological response to antigenic stimulation [80]. In earlier studies, *Klebsiella* and *E.coli* strains have been detected in human stool samples and these strains were

shown to interact with the human anti-α-Gal antibodies thus supporting the hypothesis [80]. However, two upcoming studies have challenged this hypothesis as they couldn't detect α-Gal on most of the gut microbes, thereby questioning source of antigenic stimulation in humans [81, 82]. The anti-α-Gal antibodies play a crucial role in rejection of xenografts of porcine or bovine origin and constitute a well-recognized immunological barrier in xenotransplantation. Hence, efforts are being made to produce α-1,3-GT KO animals (mainly pigs) for raising transgenic organs [83-85]. The glycolipids found in rabbit red blood cells are rich in α-Gal glycan; when injected into solid tumors these glycolipids are presented onto tumor cells, which leads to complement-mediated lysis of the tumor cells in the presence of anti-α-Gal antibodies [86]. Additionally, a systemic anti-tumor response was observed in GGTA1 KO mouse model upon injection of glycolipids into the melanoma tumor making anti-α-Gal antibodies an interesting candidate for anti-cancer treatment [87].

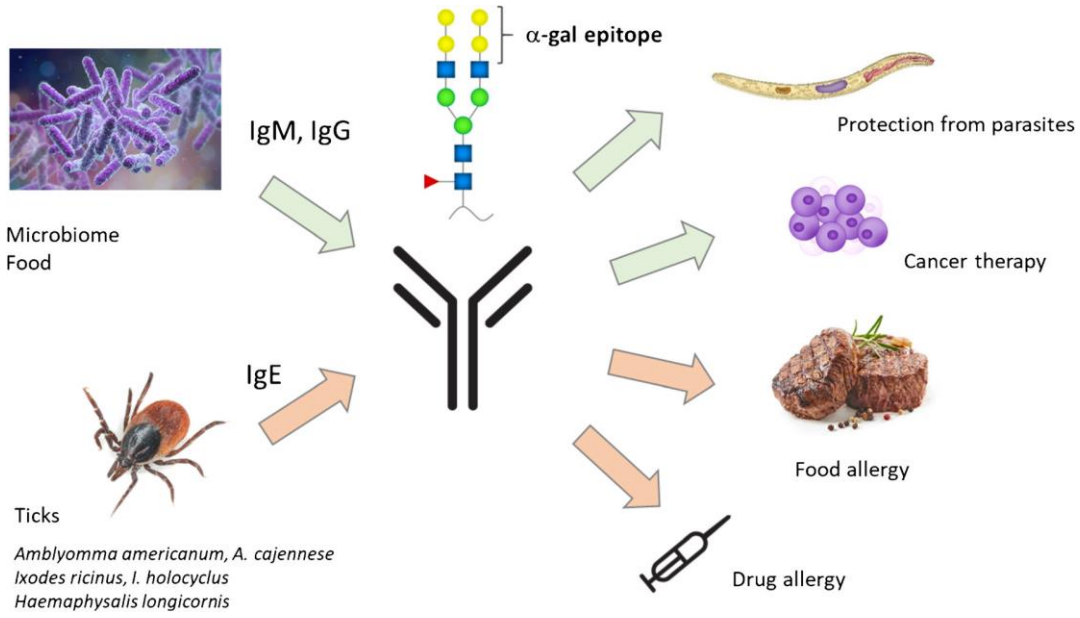

Figure 2. Diverse role of the anti-α-Gal antibody – IgG and IgM are believed to be commonly induced by continuous stimulation from the gut microbiome and probably by food as well. They play a protective role in parasitic infection and have been found to be useful in therapeutic treatment of cancer. On the other hand production of IgE upon sensitization by tick bite can result in development of allergic reactions which are delayed in nature and are experienced upon ingestion of mammalian food, whereas an immediate response is observed in case of intravenous administration of drugs containing the α-Gal epitope. In figure - α-Gal epitope: yellow – galactose; green – mannose; blue – N-acetylglucosamine; red – fucose. Adapted with permission from Hilger et al. [59]

2.2. IgE response to α-Gal

Contrary to IgG/IgM, IgE response against α-Gal is a relatively newly discovered phenomenon and first came to picture in 2007, when cases of immediate hypersensitivity reactions to the intravenous administration of cetuximab were observed in the southeastern region of the US [88]. Cetuximab is a chimeric monoclonal mouse-human IgG1 antibody directed against the epidermal growth factor receptor (EGFR); it is used in the treatment of patients suffering from metastatic colorectal and squamous-cell cancer. As per reports, 22% patients in the southeastern region developed hypersensitivity reactions whereas ≤3% of cancer patients from other regions of the United States developed similar reactions [88]. This observation is one of the chief studies that eventually led to

discovery of the α-Gal allergy. Following clinical observations, pre-treatment patient sera were analyzed and IgE against cetuximab were identified which were directed to the carbohydrate 'galactose-α-1,3-galactose' (α-Gal) present on the fab fragment of the mab. Since, cetuximab was produced in SP2/0 mouse cell line – known to possess active α-1,3-galactosyltransferase enzyme, the α-Gal epitope was expressed [89]. Detection of specific IgE antibodies in the pre-treatment sera pointed towards sensitization prior to the first dose of cetuximab [89]. A second observation in the same region reported an increase in the cases of delayed allergic reactions against red meat, which as per authors correlated with a general increase in population of the lone star tick '*Amblyomma americanum*' and tick-bite related incidences [90]. Most interestingly, Van Nunen and colleagues in 2009 proposed a relation between tick bites and allergy to red meat; upon observation of clinical cases of allergic reactions after consumption of red meat in 25 patients, 24 out of the 25 patients reported a history of tick bite [91] (see figure 3 'The α-Gal allergy – historical findings').

As α-Gal is commonly found in mammals, Commins et al. sought to analyze the same in patients' sera and successfully detected sIgE in the red meat allergic patients [90]. This finding coupled with clinical observation showing increase in α-Gal sIgE levels in three patients following tick bite and a correlation between IgE to α-Gal and to proteins from *Amblyomma americanum* confirmed a role of ticks in the induction of allergy against the carbohydrate galactose-α-1,3-galactose [92].

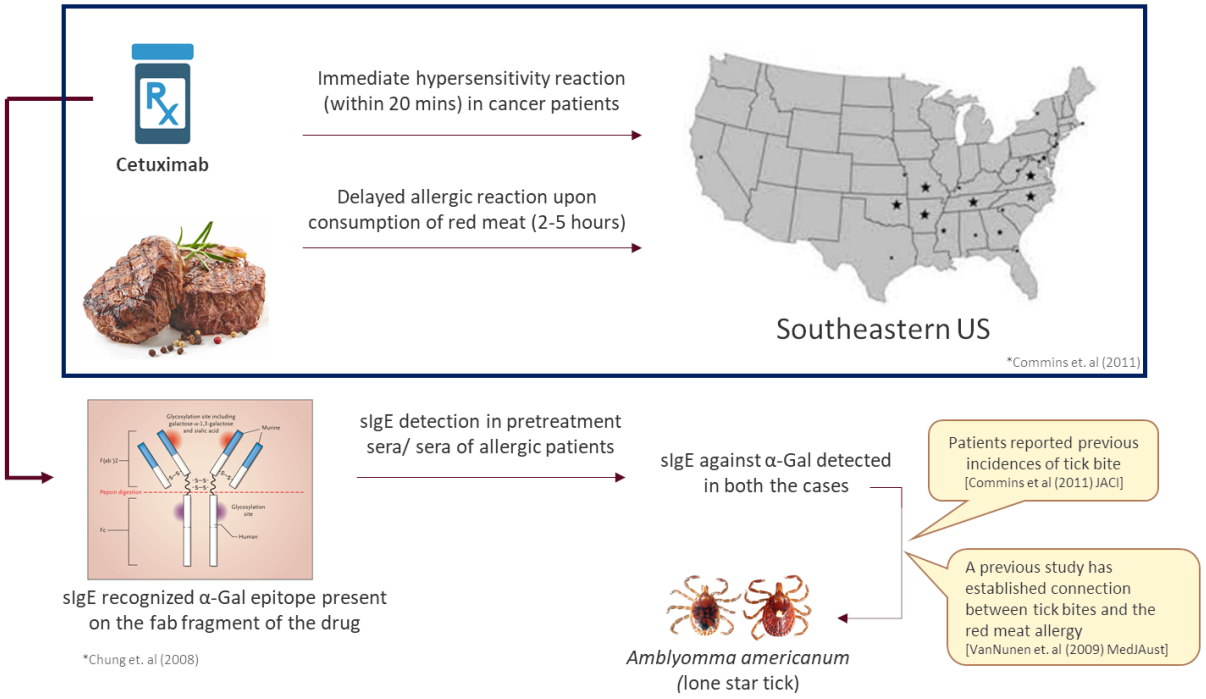

Figure 3. **The α-Gal allergy – historical findings**: Two distinct cases were observed in the southeastern region of US – first: development of immediate hypersensitivity in cancer patients upon administration of the monoclonal antibody cetuximab used in the treatment of head and neck carcinoma, second – reports of delayed allergic reaction to red meat. Both groups had IgE directed against the carbohydrate epitope α-Gal. In a previous study, the connection between tick bite and development of delayed allergy to red meat was proposed. Additional studies have confirmed the tick bite connection, thereby establishing α-Gal allergy as the first tick-borne allergy [89, 93-95]

2.3. The α-Gal allergy: epidemiology

Following studies in Australia and US, numerous cases of red meat allergy have been reported across the world. The clinical observations remain similar throughout, and the cases are most commonly reported among adults with symptoms arising within 2-6 hours after meat consumption. However, a major difference is observed in the associated tick species. Although all the reported ticks are hard-ticks, different species have been identified across different continents; moreover, more than one species have been reported on the same continent. The table below depicts a list of countries reporting clinical cases as well as identified tick species. More detail on ticks in chapter IV.

Continent	*Tick species*	*Country*
Europe	*Ixodes ricinus*	Germany
		Sweden
		Italy
		Switzerland
		Norway
		Denmark
	not identified	Belgium
	-	France
	-	Netherlands
	-	UK
	Rhipicephalus bursa	Spain
America	*Amblyomma americanum, Ixodes scapularis*	US
	Amblyomma sculptum	Brazil
	Amblyomma cajennese	Costa Rica
	"	Panama
Asia	*Amblyomma testudinarium*	Japan
	Haemaphysalis longicornis	Korea
Australia	*Ixodes holocylus, Ixodes australiensis*	Australia
Africa	*Amblyomma herbraeum*	South Africa
	Amblyomma variegatum	Ivory coast
	not identified	Zimbabwe

Table I. Case reports of α-Gal allergy from across the world [55, 96-98]

Sensitization to α-Gal

Due to the association of red meat allergy with tick bites, populations at a higher frequency of tick exposure are also at a greater risk of being sensitized. Several studies on populations highly exposed to tick bites have shown an increased frequency of sensitization to α-Gal compared to the populations at a lower rate of exposure.

Occupational exposure: Increased tick exposure in occupations that require spending considerable amount of time outdoors, especially so in locations with higher chances of a tick encounter can significantly increase risk of sensitization. In 2017, Fischer et al. reported the prevalence of sIgE in a cohort of forest workers and found 35% to be sensitized (sIgE ≥0.1 kU/L) [99]. In Spain, 15% prevalence rate (sIgE ≥0.1 kU/L) to α-Gal sIgE was reported in a high-risk population of forest workers compared to 4% prevalence in control population (not recalling tick bites) [100]. In US, Bellamy et al. (2021) observed 40% sensitization prevalence (sIgE ≥0.1 kU/L) among Kentucky timber harvesters, and forestry and wildlife practitioners [101]. When compared to observations in urban settings forest workers are at a higher risk and thus should be more cautious and take necessary precautions to avoid tick bites.

Geographical location can be an indicator of higher sensitization when taking into consideration regions more conducive to tick survival versus colder climate or areas with less vegetation. As observed by Villalta et al. 24.7% of population in a rural pre-alps area in Italy were α-Gal sIgE positive compared to 1.2% of the urban population [102]. Similarly, populations less susceptible to tick bites report a lower prevalence as seen in a cross-sectional study of randomly selected general adult population from Denmark and Spain, which shows sIgE prevalence rate of 5.5% and 8.1% (≥0.1 kU/L) respectively [103]. In southeastern US, prevalence rate was found to vary from 15% to 25% as observed in a cohort of 50 – 250 patients [88, 104]. As a general observation, occupational exposure as well as outdoor activity and rural settings resulted in higher sensitization rates. Another interesting case is that of tick endemic regions, where an increased prevalence of α-Gal sIgE can be expected due to higher a risk of bites. Study by Tjernberg et al. (2017) further supports this claim, they found a prevalence rate of 14% of sIgE to α-Gal in blood donors from a highly Lyme borreliosis endemic region in Sweden [105]. In conclusion, several factors, all related to increased chances of exposure to tick bite have been found to play a major role in increased sensitization prevalence among different populations.

Allergic individuals: Another group experiencing an increased risk of sensitization is allergic patients; a prevalence rate of 19.9% in allergic patients and 30.2% in insect venom allergic patients (sIgE ≥0.1 kU/L) have been reported [106]. In a study exploring significance of location in allergic patients Mateo-Borrega and colleagues (2019) recruited adults presenting with urticaria or anaphylaxis from 14 Spanish locations. They report a prevalence rate (sIgE ≥0.35 kU/L) of 37.68% in rural, 15.38% in semi-urban, and 7.85% in urban population while 2.4% in the control group which had allergic symptoms to foods other than red meat [107].

2.4. Risk factors

Several risk factors have been identified as contributing to the red meat allergy. These can be divided into two categories – factors leading to increased chances of sensitization in healthy individuals and factors contributing to the onset of symptoms in already allergic individuals. With respect to sensitization, most important factor is an increased exposure to tick bites, which as discussed above can be due to exposure during an outdoor activity like hunting and in occupations such as forest work, during sports activity, and in leisure time spent outside, etc. [99-103]. In relation to increased tick

exposure another risk factor is living in areas with increased tick infestation which can be either a tick endemic region or due to tick-favouring condition such as increased vegetation and a warm climate. Additionally, male sex have been proposed as a risk factors as higher percentage of the patients observed were males, however it relates more to a lifestyle preference of an individual rather than an actual risk [108]. A second set of factors leading to an increased risk of allergic onset include co-factors such as – physical exercise, alcohol, NSAIDs, etc. which have been found to decrease the reaction time and increase symptom severity in some patients [58].

2.5. Clinical manifestation

α-Gal allergy is now termed a 'syndrome' due to the multi-faceted development of the clinical responses. A distinctive feature of this allergy as previously described is a delayed onset of systemic reactions typically 2 to 6 hours after consumption of mammalian meat, setting it apart from most IgE-mediated food allergies where reactions occur within minutes to an hour of food consumption [59]. However, patients can develop immediate hypersensitivity reactions upon parenteral administration of drugs and biologicals containing α-Gal from a mammalian source e.g. cetuximab [109-112]. Allergen stability such as factors involved in digestion, absorption, and presentation of the allergen to the immune cells may play a role. Delayed reactions are frequently observed upon pork and beef muscle meat consumption, but also deer or lamb; on the other hand, consumption of pork kidney and mammalian innards often result in a more severe and rapid development of clinical symptoms, appearing within 2 hours of meat consumption [113]. In addition, co-factors were shown to influence the severity and rapidity of the reaction onset by shortening the response time and by increasing the gastrointestinal absorption [58]. Recently, α-Gal-carrying glycolipids were postulated to be responsible for the delayed occurrence of symptoms, due to their in general slower digestion kinetics and late appearance in the blood circulation [114].

Red meat allergy is predominantly reported in adults and onset is usually observed after age 40. As many as 58% cases of α-Gal allergy were observed after the age of 40, in a study on 261 subjects by Wilson et al. [115]. Most studies report no obvious role of atopic disposition, although some studies do describe an impact of age as well as atopy in the disease development. The most commonly reported symptoms include cutaneous manifestations such as urticaria, angioedema, pruritus etc. [60]. Study on a cohort of α-Gal patients reported 90% with urticaria, 74% with gastrointestinal symptoms and 50% with anaphylaxis [116]. However, many recent studies have reported gastrointestinal symptoms both isolated and together with skin reactions, Wilson et al. report GI symptoms in 64% of patients and Mabelane et al. report isolated GI symptoms in 20% of the cases [115, 117]. Recently, Richards and colleagues confirm presence of α-Gal IgE in patients experiencing abdominal pain and diarrhea but without classical symptoms like urticaria, pruritus or anaphylaxis, further confirming findings by Croglio et al. in patients with isolated GI symptoms [118, 119]. Several patients reported an eventual diminishing of symptoms upon elimination of mammalian meat from diet.

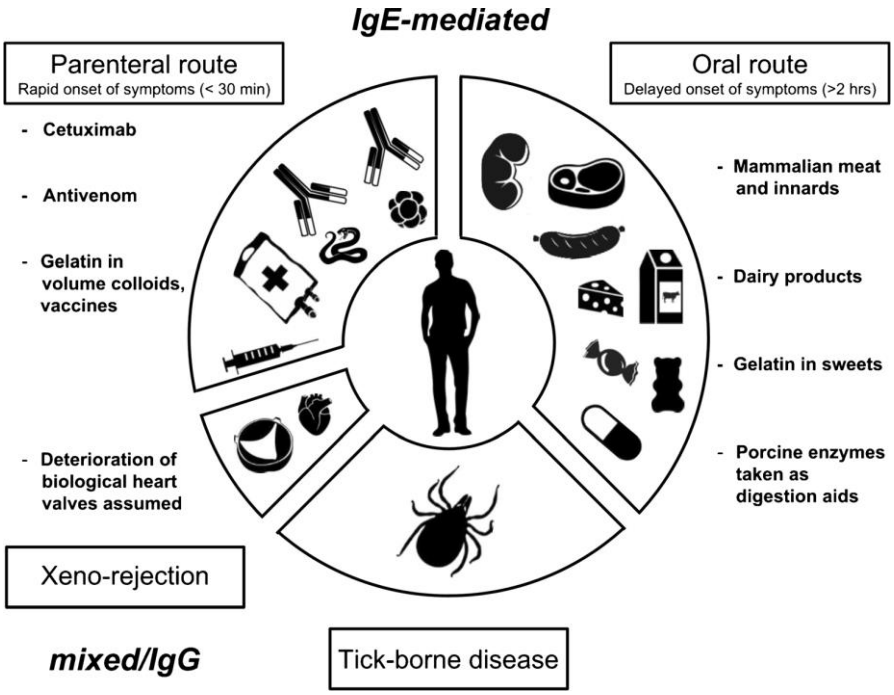

Figure 4. **The α-Gal syndrome**: describes an IgE-mediated allergy to the carbohydrate galactose-α-1,3-galactose (α-Gal). It is first allergy to be characterized as a tick-borne disease that results in development of allergic response towards mammalian meat and mammalian-origin products. Antibodies against α-Gal (mixed/IgG) are known to play role rejection of xeno-grafts, the manifestation of allergic reaction occurs in two flavours – delayed when allergen source is encountered via oral route, immediate upon encounter of the allergen via parenteral route. Adapted with permission from Hilger et al. [59]

2.6. α-Gal in food and non-food sources

A delayed onset of reactions is often reported upon consumption of mammalian meat (beef, pork, lamb etc.), dairy products such as milk, cheese, cream, ice-cream and food items like burgers, Bolognese [59]. Clinical cases from Europe report severe reaction with early onset upon consumption of innards such as kidney, lungs, sweetbreads, tripe etc. Some reports suggest reactions in patients upon consumption of food cooked on pan with leftover beef/pork grease and chicken sausage with pork casing [120]. A strong reaction to pork kidney occurring within 1-2 hours of consumption have been reported and our group has previously identified two heat resistant α-Gal-carrying proteins aminopeptidase N and angiotensin-I-converting enzyme [121]. Several α-Gal-carrying proteins were previously identified by Apostolovic and colleagues [122], in addition two beef proteins – Laminin γ-1 and collagen α-1 (VI) chain were reported to carry α-Gal in a previous study [123]. Although tolerated by most patients, milk can be a source discomfort in some patients, recently 3 proteins namely bovine γ-globin, lactoferrin, and lactoperoxidase were identified that were recognized by patient IgE [124]. The α-Gal epitope is a major barrier in xenotransplantation and many studies have characterized α-Gal on glycolipids in mammalian tissues and organs, chiefly pork and babbon organs and kidney from rabbit, sheep, rat, and pig [125-127]. The high abundance of α-Gal in pork kidney makes it a potent trigger of allergic reactions [128].

Due to natural occurrence in mammals, α-Gal is a trigger of allergic reactions in various non-food sources as well. Most important one being biologicals derived from a mammalian source – such as monoclonal antibodies expressed in cell lines with active α-1,3-galactosyltransferase activity, drugs of porcine origin Enzynorm f and Creon have been found to react with patient IgE [129] and thus careful

usage is advised. Anti-venoms are mostly produced by immunization of sheep and horse, enzymatic digestion of anti-venom formulations (composed of polyclonal IgG antibodies) can expose hidden α-Gal epitope on Fab fragments, which has been shown to be a source of hypersensitivity reaction in α-Gal allergic patients [109, 130]. Porcine and bovine derived heparin if not treated to remove α-Gal during manufacturing can put allergic patients at risk; prosthetic heart valve (porcine/bovine) are treated with glutaraldehyde to increase biocompatibility, study by Naso and colleagues detected presence of uncovered epitope making these valves a source of potential risk. In addition, premature degradation of bioprosthetic aortic valve is postulated based upon observation in two α-Gal allergic patients [131]. Moreover, an increased risk of inflammation in response to α-Gal glycolipids is proposed in allergic patients; however, these postulations point more towards possible risks and there is still lack of clinical studies showing actual involved risk [132]. Lastly, gelatin has come across as a major concern due to its wide usage both in food and pharma industry. α-Gal epitope has been detected in gelatin colloids as well as on collagen α-1 (VI) chain [123, 133]. Gelatin is widely used in vaccines and in colloidal form as plasma expander and many cases of allergic reactions to gelatin-containing vaccines such as zoster and mumps/measles etc. have been reported [134-137] and warrants caution.

2.7. Clinical management

Diagnosis is not straightforward as the time of food consumption and emergence of symptoms lie hours apart and patients seldom make a causative link between food ingestion and symptoms. Involvement of co-factors and variation in the time of symptom appearance can further complicate diagnosis. Therefore, careful examination of clinical history is an important aspect of the diagnosis especially a history of tick bites. Most common methods include skin prick tests and serum IgE analyses to determine specific IgE to α-Gal, beef, and pork and to cat albumin and milk as well [60, 120]. α-Gal sIgE levels ≥2 kU/L or >2% of the total IgE are strong predictors of clinically relevant diagnosis [120]; moreover a successful resolution of symptoms upon red meat avoidance can further confirm the diagnosis. It can be difficult for patients to eliminate α-Gal-containing food products completely due to it occurrence in a wide range of food including dairy, however most patients are able to tolerate some meat and clinically react to >20 g of meat [50] and thus can be advised to continue eating small quantities to maintain tolerance. Moreover, 80% of cases were found to tolerate dairy products including milk and therefore avoidance of dairy is often not advised and should be eliminated only in cases of clinical reactivity [120]. Most importantly further tick bites should be avoided as it can sustain or enhance α-Gal IgE levels in patients.

Skin prick tests using commercial extracts as well as prick-to-prick test with beef or pork meat were found to yield weak or false-negative results and can present difficulty in correct diagnosis due to low abundance of α-Gal in meat extract. Prick-to-prick tests using pork or beef kidney are more sensitive and hence recommended in clinical practice, in some cases intradermal testing using gelatin can be employed [138]. Oral food challenges (OFC) are still the gold standard but their use in α-Gal allergy is not recommended due to the delayed occurrence of symptoms which can be difficult to manage and can cause severe anaphylactic episodes. Thus, OFC should only be performed in case the diagnosis is unclear or patient shows variable response to meat exposure such as reactivity upon involvement of co-factors in an otherwise sustained tolerance, such patients can continue eating small quantities of meat and avoid co-factors [50].

The commercial assays are still the most reliable for quantitative measurement of serum IgE, however the accurate determination depends upon the binding capacity of the antigen-specific IgE. Currently, bovine thyroglobulin which is a natural α-Gal-carrying protein is used in routine ImmunoCAP for

determination of sIgE values (threshold >0.10 kUA/L) (ThermoFischer guidelines). Although highly accurate in determination of sIgE titers, it can't distinguish symptomatic individuals from the asymptomatic sensitization. Mehlich et al. have shown efficacy of Basophil Activation Test in identifying clinically relevant cases of α-Gal allergy, due to higher sensitivity and basophil reactivity in patients compared to the sensitized individuals [139] thereby establishing use of BAT assays in analyzing clinically relevant cases of α-Gal allergy.

Basophil Activation Test

Basophils are the least abundant granulocyte circulating in blood with a population of approximately 0.5% of the leukocytes; they play a key role in immediate hypersensitivity reactions. Specific IgE is present bound to the high affinity IgE receptor (FcεRI) on the surface of basophils in allergic patients. Upon contact with allergen and cross-linking of IgE, basophils degranulate and release mediators such as histamines, which leads to development of allergic symptoms. Basophil activation test (BAT) is an *in vitro* simulation of the *in vivo* reaction occurring in patients. It has emerged as a substitute to oral food challenge (OFC) and has been shown to eliminate the need of oral food challenge, for example in case of peanut allergy as many as 60-80% cases can be accurately identified without a need for OFC [140]. However, BAT is not a routine test and is performed in a laboratory instead of a clinical setting. It can be used in addition to routine tests such as skin prick test (SPT) or IgE measurement in case of an inconclusive result.

For BAT assay, patient's whole blood is incubated with varying quantity of allergen either as an extract or in isolated form. The allergen mimics *in vivo* reaction and leads to activation of basophils upon binding and cross-linking of the surface bound specific IgE. In activated basophils, intracellular granules bearing CD63 protein fuse with the cellular membrane and are therefore exposed [50]. Staining reagents are added, monoclonal antibodies (specific for the cell surface markers) with fluorescent tag, and after removing erythrocytes samples are analyzed in flow cytometry [140]. For basophil identification, after gating for singlets basophils are gated using specific cell surface markers such as CD123, CD193 (CCR3), or CD203c, and activated basophils are identified with the CD63 markers (figure 5 shows gating strategy used in chapter III). Upregulation of CD63 is strongly correlated with the histamine release (Bühlmann BAT assay) and therefore can give an insight into the severity of clinic response in patients. Various parameters are analyzed such as – dose-response curve, EC50 (half-maximal effective concentration), area under the curve etc. to determine basophil reactivity and specificity. However, basophils from some patients (5-10%) are unresponsive and therefore the assay result is inconclusive in these patients [141].

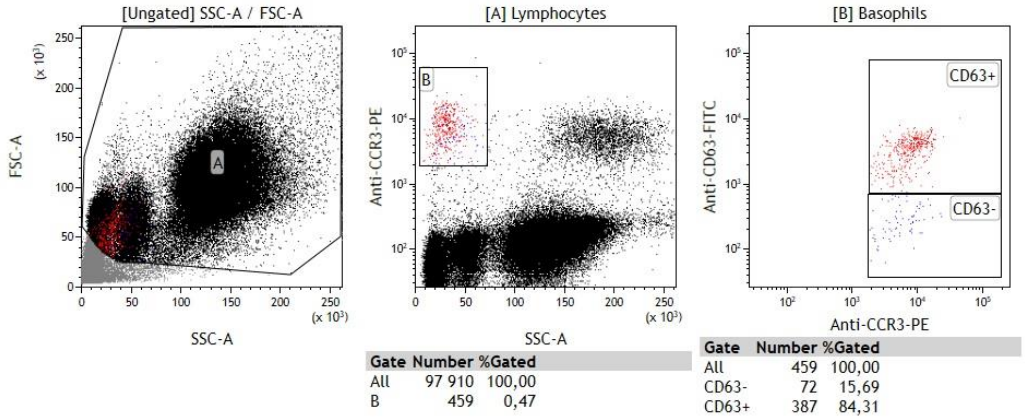

Figure 5. **Basophil Activation Test**: gating strategy (SSC^{low}, $CCR3^{+}$ -> $CCR3^{+}$, $CD63^{+/-}$) for BAT assays included in chapter III of the thesis – FSC–SSC plot to select single cell events, $CCR3^{+}$–SSC^{low} identifies basophils, and $CD63^{+}$–$CCR3^{+}$ double positive identifies activated basophils. Positive control with anti-FcεRI shown in BAT assay using whole blood from an α-Gal-allergic patient. Adapted from Chakrapani et al. [142]

Significance of BAT: IgE levels often fail to represent clinical responsiveness of a patient, irrespective of high IgE levels, a patient can exhibit tolerance against consumed food whereas in other cases even small quantities of serum IgE can result in onset of severe reactions. Basophil activation test is highly accurate at predicting clinical outcome and therefore it can be performed in precedence to oral food challenge if required. Additionally, it can serve as a complementary test to skin prick test in order to avoid misdiagnosis in case of a false positive result. It can also be used to determine IgE reactivity against recombinant allergens which can't be performed in individuals. BAT however requires a specialized research setting and therefore can't be used in routine. Taken together with the clinical history and IgE measurement BAT can improve the accuracy of allergy diagnosis [50, 140, 141].

In FA, it is used to analyze clinically relevant cases along with patients' clinical history, skin prick test results, as well as IgE measurement. In addition to diagnosis, BAT assays are also useful in monitoring successful resolution of allergy following immunotherapy, improvement upon treatment with biologicals or natural resolution of symptoms over time [140].

2.8. The α-Gal epitope

The α-Gal is a terminal disaccharide structure, galactose-α-1,3-galactose, and it is present linked to the N-acetylglucosamine (GlcNAc) on glycoproteins and glycolipids. The complete glycan structure is represented as 'Galα-1,3-Galβ-1,4-GlcNAc-R'. All mammals express α-Gal except old world monkeys, apes and humans due to the loss of ability to express α-Gal because of a frameshift mutation in the gene (GGTA1). The galactose sugar from UDP-Gal as a donor is transferred onto N-acetyllactosaminyl group (Galβ1-4GlcNAc-R) via α-1,3-galactosyltransferase enzyme which is encoded by the α-1,3GT gene (GGTA1) and is active in the trans-Golgi apparatus [67]. This results in formation of the α-Gal epitope – Galα-1,3-Galβ-1,4-GlcNAc-R, which can be linked onto protein via N-linkage and onto lipids via O-linkage.

In glycoproteins, glycans are linked to the protein structure by either N- or O-linkage. N-linkage refers to attachment of the glycan N-acetylglusamine (GlcNAc) to the nitrogen atom of Asn side chain by a β-1N linkage in the consensus motif "Asn-X-Ser/Thr" (X denotes any amino acid except proline) on the protein structure [143]. Whereas, O-linkage refers to attachment of glycan core to the oxygen atom of the hydroxyl group on amino acids serine or threonine. O-linkages are most commonly present as GalNAc and GlcNAc. GalNAc are present in abundance on the extracellular and secreted glycoproteins these glycoproteins are mostly mucin-type O-glycans. On the other hand, O-linkages on GlcNAc are not added in the Golgi apparatus, but are synthesized by O-GlcNAc transferases that typically occur in cytosol and nucleus (Reily 2019, nature) and therefore it's highly likely that α-Gal onto O-linkage is not present or is perhaps difficult to characterize.

Glycosphingolipids are sphingolipids with glycans attached at the hydroxyl position of the ceramide; α-Gal can be transferred to the GlcNAc residue of the attached glycan by the active α-1,3GT gene thus resulting in α-Gal-carrying glycolipids [144, 145]. Several α-Gal-like structures exist due to the activity of glycosyltransferases belonging to the GT6 family –

The glycosyltransferase family – GT6

Glycosyltrasferases are intracellular membrane-bound enzymes that participate in synthesis of glycan structures on glycoproteins and glycolipids. Enzymes of the glycosyltransferase 6 (GT6) family catalyse transfer of galactose (Gal) or N-acetylgalactosamine (GalNAc) on α-1-3-linkage in various structures [146]. They participate in the synthesis of A and B blood group antigens (ABO gene; AT/BT enzymes), the α-Gal epitope (GGTA1 gene; α-1,3-GalT enzyme), Forssman antigen (GBGT1 gene; FS enzyme), and iGb3 glycolipid (A3GALT2 gene; iGb3S enzyme) [147]. GT6 homologs are present in mammals, birds, fish, and amphibians; however, in humans all GT6 genes except ABO are pseudogenes [148]. The ABO antigens are membrane bound glycoproteins and glycolipids commonly occurring on erythrocytes as well as on various epithelial/endothelial cells. A glycan which forms the blood group A is generated by α-1,3GalNAcT (A3GALNT) encoded by *A* allele whereas *B* allele encodes α-1,3-GalT (A3GALT1) forming the B glycan on blood group B. The Forssman antigen (globopentosylceramide) is a glycolipid containing terminal α-1,3GalNAc, which is transferred by α-1,3GalNAcT GBGT1 – a gene with similar sequence as ABO transferases (ref. essentials of GB ch14). Whereas, iGb3S is known to synthesize α-Gal epitope on lactose containing lipid isoglobotrihexosylceramide (iGb3) in mammals [149]. In conclusion, Forssman antigen exhibits the same GalNAc specificity as *A* transferase in blood group A whereas α-1,3GalT and iGb3 show same specificity to galactose as B transferase. Only difference being

presence of fucosylation on A and B blood group glycan, which is absent in the glycan structure of the other three antigens [150].

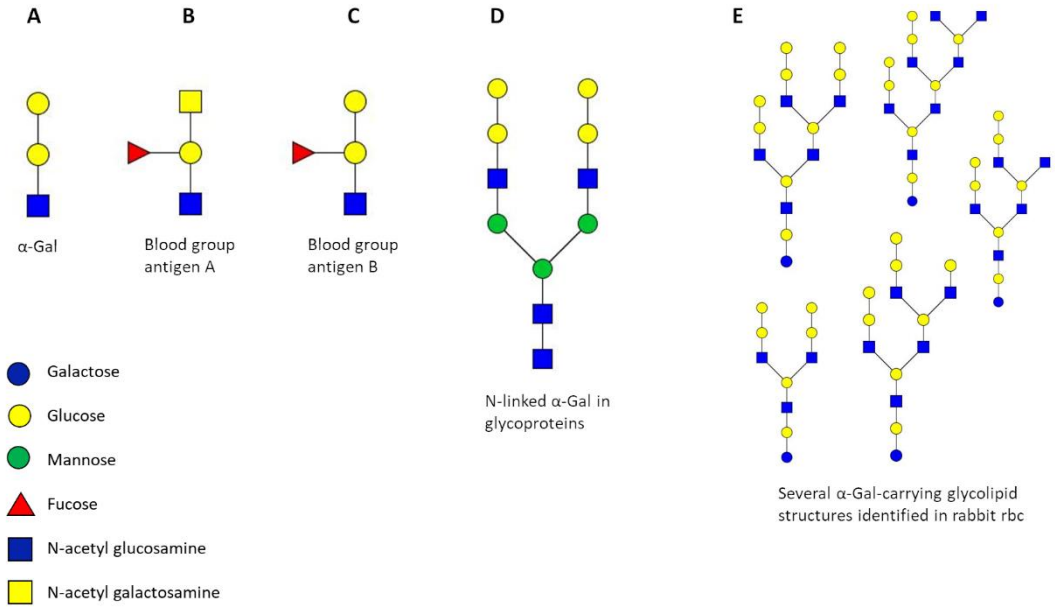

Figure 6. **Glycan structures** – A. the α-Gal epitope, B. A blood group antigen, C. B blood group antigens, D. N-linked α-Gal in glycoproteins, E. α-Gal-carrying glycolipids identified in the rabbit erythrocytes extract (glycan chain is attached to a ceramide at O-linkage) [143]

Antigen resemblance in population with B-blood group

Blood group B antigen (Gal-α-1,3(Fuc-α-1,2)-Gal) is structurally similar to the α-Gal epitope and possess the similar terminal disaccharide structure along with an additional fucose residue which is connected to the galactose on glycan core by α-1,2-glycosidic linkage (figure 6). Therefore, individuals with blood group B produce lesser quantities of anti-α-Gal antibodies due to increased self-tolerance [151] however they're highly specific. The anti-α-Gal antibodies from subjects lacking B antigen (A/O blood group) react to both the α-Gal epitope and the B antigen, whereas the α-Gal IgG antibodies in donors of blood group B bind solely to the α-Gal epitope. Due to this increased specificity blood group B individuals exhibit a significantly reduced antibody response to α-Gal which can confer a protective/detrimental response depending upon the situation [152, 153]. Cabezas-Cruz and colleagues report higher incidences of malaria and tuberculosis among B blood group in endemic regions, and attribute it to presence of less anti-Gal antibodies within the B blood group population compared to A/O blood groups. On the other hand, this structural similarity is shown to result in lower risk of sensitization to α-Gal in individuals with blood group B due to increased tolerance towards the α-Gal epitope [154]. It is a highly interesting finding and should be explored further, especially so, as studies by Fischer et al. and Mateo-Borrega et al. report observing no significant difference in the distribution of allergic cases among different blood groups [107].

Specificity of anti-α-Gal antibodies

Initially, anti-α-Gal antibodies were thought to be universally specific for the terminal disaccharide structure Gal-α-(1,3)-Gal, however studies later found as much as 10 times lower affinity of anti-Gal from human serum towards disaccharide compared to the trisaccharide [155]. Mckane et al. proposed that the composition and steric arrangement of glycans, particularly those containing a Glc or a GlcNAc in the third position, may affect the binding of these antibodies [156]. This finding was confirmed in another study, where authors could show a greater inhibition of human serum by lengthening the carbohydrate thereby pointing towards a role of the third monosaccharide in increased specificity [157]. Moreover, by analysing the reactivity of several monoclonal antibodies (mAb) directed against α-Gal obtained from α-1,3-GT-KO mouse immunized with rabbit red blood cells Milland et al. reached to a similar conclusion. They could successfully show that the mAb can distinguish between glucose (Glc) and N-acetylglucosamine (GlcNAc) i.e. the glycan structure present in the third position of the α-Gal epitope [149]. Therefore, the role of the third glycan in determining specificity is well established, however the terminal disaccharide still appears to play a major role in determining anti-α-Gal binding. In any case, natural anti-Gal antibodies in healthy individuals overall exhibit a low specificity and can bind non-specifically to other glycan epitopes too [158]. In the context of the α-Gal binding, Plum et al. used a monoclonal M86-based IgE and polyclonal antibodies from human serum to characterize binding with a glycan structure- Galα-1,3-GalβOMe, the recognition of the complete disaccharide was successfully shown [159]. A more recent study resolved crystal structure of M86 binding with the α-Gal glycan (α-1,3-galactobiose) and confirmed previous observations. They further resolved structures of IgM and IgG isotypes from humans and found similar recognition pattern as M86. Therefore, emphasizing solely the role of disaccharide in recognition by anti-α-Gal antibodies [160].

III. Immune response to carbohydrates

Traditionally, carbohydrates were considered to be of low immunological significance due to their inability to activate T-cells and were largely considered to induce a T-cell independent immune response. Therefore, it was accepted that anti-carbohydrate antibodies exhibit low-affinity and specificity and chiefly belong to the isotype IgG2 in the blood. However, this view has changed in recent years with the observation of anti-carbohydrate antibodies of multiple subclasses of immunoglobulins and presence of high-affinity antibodies against glycans [161]. Studies have confirmed a role of T-cell dependent activation when carbohydrates are present in conjugation with proteins and lipids in the form of glycoproteins or glycolipids. Thereby showing that the carbohydrates can give rise to both T-cell independent (polysaccharides) and T-cell dependent responses (glycoproteins and glycolipids) depending upon there structure. In addition to the adaptive response, innate immune response and the gut antigenic stimulation can induce production of IgG and IgA antibodies, which are already known to play an important role in protection against pathogens [76, 162-164].

3.1. T-cell-independent response

Antibodies produced without an active vaccination are often termed as natural antibodies and a large portion of these are anti-carbohydrate antibodies [67]. However, in truest sense natural antibodies are the ones which are naturally present without any kind of stimulation, they're therefore poly-reactive. However, sometimes antibodies produced via antigenic stimulation in gut are considered as natural antibodies as well. They bind with low affinity to a wide range of carbohydrates. An early life colonization by gut microbes presents varied carbohydrates to the immune system, leading to the production of anti-glycan antibodies [64]. These antibodies were believed to be of low-affinity and specificity, and chiefly belonging to the subclass IgG2 [165].

T-cell independent activation of immune system by carbohydrates can occur upon encounter of two types of antigens – type 1 comprises bacterial components that can initiate polyclonal B-cell induction due to their heterogeneous structure, and type 2 antigens such as polysaccharides can cross-link B-cell receptors due to the presence of repetitive motifs and thus result in cell activation [162, 163]. Additional signals like toll-like receptors when triggered by antigens can result in production of low-affinity IgM and IgG antibodies. In the mucosal immune system, the BAFF/APRIL pathway is involved in the production of glycan-specific IgA and IgG upon microbial encounter in the gut [161]. Commonly occurring carbohydrate antibodies in humans recognize the glycans – α-Rhamnose, N-glycolylneuraminic acid (NeuGc), ABO groups, α-Gal, forssman, lewis and P antigen among others and constitute the highest titer of anti-carbohydrate antibodies [161].

3.2. T-cell-dependent response

Several studies have shown that carbohydrates can be presented in a T-cell-dependent manner and take part in the adaptive immune response. Studies using glycan-arrays have detected multiple subclasses of these antibodies and have found many of them to be of high-affinity, which is normally the case only in the presence of T-cell help [166]. Thereby, showing significant involved of T-cell help, however T-cell dependent presentation is only possible in case of conjugation with a carrier molecule and can't occur in the presence of an isolated glycan. Requirement of a carrier protein for T-cell induction makes it difficult to obtain vaccine candidates for glycans. Common carbohydrate-derived

antigens include – glycopeptides from processing of glycoproteins, glycolipids bound to CD1d, zwitterionic polysacharride capable of activating T cells and glycoconjugate vaccines.

T-cell-dependent carbohydrate antibody production involves classic steps i.e. uptake of the antigen by antigen presenting cells (APCs) followed by processing with the help of proteases, glycosidases etc. and presentation in conjugation with peptides on the cell surface by the major histocompatibility complex (MHC). Finally, these cell-surface bound antigens are recognized by T-cell receptors leading to activation. Antigens can be processed and presented by APCs via two pathways – endogenous pathway where antigens in the form of glycopeptides are presented onto MHC I molecules via cytosolic pathway, and exogenous pathway where glycopeptide antigens are presented onto MHC II molecules via endocytic pathway. CD8+ T-cells recognize the MHC I complex whereas MHC II complex binds to the CD4+ T-cells [162-164]. Common T-cell-dependent carbohydrate presentations are –

Glycopeptides and glycoproteins

Numerous studies have now shown an important role of peptide glycosylations in T-cell activation upon presentation via MHCI/II molecules, which depends upon size and structure of the carbohydrate epitope as well as its complexity and spatial arrangement on the peptide. In one of the very first study, authors used synthetic glycopeptides and T-cell hybridomas to investigate antigenic properties of a carbohydrate epitope [167]. They conjugated disaccharide galactobiose to the lysozyme peptide from hen egg-white and used it as an antigen and produced T-cell hybridomas. These hybridomas were specific to the glycan as well as to the peptide sequence and couldn't be stimulate using another glycopeptide sequence [168]. Studies have confirmed similar findings of glycopeptide specific responses of the T-cells [164]. In addition, monosaccharides have been found to have higher binding affinity to MHC II compared to complex glycan structures and were more specific compared to T-cell directed towards complex heavily glycosylated peptides which showed increased cross-reactivity [169]. Glycosylation site is an important factor contributing to the T-cell recognition, and glycans on distant locations from the amino acid that anchors to MHC II as well as glycans present linked to the anchoring amino acid resulted in no T-cell activation [170]. Hence, the glycans when conjugated to peptides/proteins were able to stimulate T-cell only when present outside the MHC II attachment site, pointing towards a major role of the glycan site. Studies have confirmed similar findings on MHC I presentation as well as with respect to recognition of the O-linked glycans, however discussion on O-linked glycans is beyond the scope of this thesis.

Natural exposure to glycoproteins can activate T-cells; these glycoproteins can be processed in two ways resulting in – peptides devoid of glycans or in peptide fragments carrying glycans that has survived antigen processing. These shorter glycosylated peptides resulting from processing of the exogenous proteins by APCs are presented by MHC II receptors and bind to the CD4+ T-cells [167]. The glycoprotein allergen phospholipase A2 originating from the bee venom is known to induce a glycan-specific activation of CD4+ T-cells. In further investigation, these T-cell clones were shown to respond only to the native phospholipase allergen and not to variants such as only the glycan motif, deglycosylated proteins and a mixture of both [171]. Thus clearly showing role of glycans on protein structures in T-cell activation. A second major study supporting direct recognition of the glycans came from glycosylated peptides of type II collagen. These glycosylations were shown to play a major role in collagen-induced arthritis and a reduction in disease severity was observed upon removal of glycosylations due to diminished T-cell activation [172, 173]. In addition, various studies have clearly shown involvement of cell surface glycoproteins on bacteria in CD4+ T-cell activation upon their

processing in APC and presentation via MHCII [164]; the T-cell reponse were impaired upon treatment by glycosidase or protein production in E.coli [174].

Zwitterionic polysaccharides

Zwitterionic polysaccharides (ZPSs) are glycans with alternating positively and negatively charged monosaccharides; they are processed by oxidative reactions within antigen-presenting cells via MHC II pathway and presented to CD4+ T-cells. The zwitterionic motif facilitates MHC II binding through electrostatic interactions. Most polysaccharides on bacterial cell-surface are either negatively charged or neutral, and although they undergo similar endosomal processing and presentation as ZPSs, they fail to bind MHC II, hence normally favouring a T-cell independent carbohydrate immune response. Polysaccharide A (PSA) expressed on gram-negative bacteria such as *Bacteroides fragilis*, is most widely studied zwitterionic polysachharides known followed by *Staphylococcus aureus* (CP5, CP8) and *S. pneumonie* (Sp1) [162, 175-177].

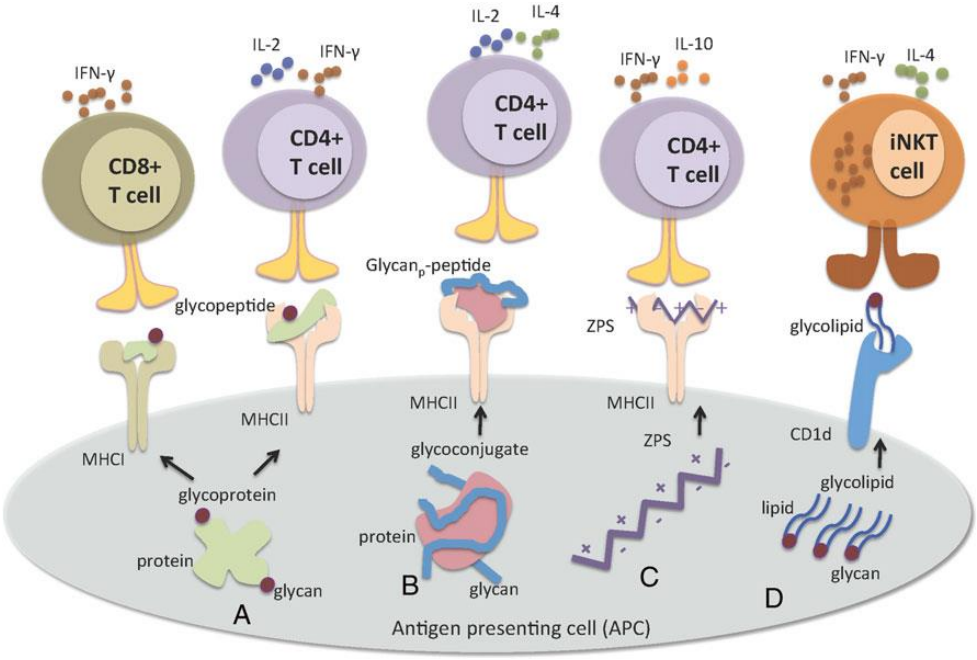

Figure 7. **T-cell dependent immune response of the carbohydrate antigens**: A. Glycopeptides: resulting from processing of glycoproteins by antigen-presenting cells or synthetically prepared are presented to CD8+ or CD4+ T-cells by MHCI and MHCII molecules. B. Glycoconjugates: conjugates of capsular polysaccharides with carrier proteins are processed by proteases and by reactive oxygen species (ROS) in APCs into polyglycan-peptides, binding of peptide backbone to MHCII helps in presentation of the glycan epitope to CD4+ T-cells. Upon activation of glycan-specific T-cells cytokines such as IL2 and IL4 are produced. C. Zwitterionic polysaccharide: reactive nitrogen species in APCs process extracellular zwitterionic polysaccharides into smaller polysaccharides that can be presented onto the surface of APCs for recognition by T-cells. D. Glycolipids: The CD1d molecules that are similar to the MHCI present glycolipids to the iNKTs; iNKTs in response produce cytokines IL4 and IFN-γ. Reproduced from Sun et al. [164]

Glycolipids - iNKT cells

iNKT cells are thymus-derived T cells exhibiting co-expression of T-cell receptors along with the typical NK cell receptors. The iNKT receptor is composed of invariant Vα24Jα18/Vβ11 in humans, which recognizes glycolipids presented by the MHC class I like CD1d molecules leading to secretion of Th1 or

Th2 polarizing cytokines, depending upon the glycolipid molecule [178]. They are a part of the innate immune system, lead to shaping of the adaptive immune response thereby acting as a bridge between the two, and play a key-role in tumor immunity, autoimmunity, infection [178]. In addition, iNKTs play an important role in allergies related to glycolipids, phospholipids and lipids in general [179], glycolipids presented by DCs can induce iNKT cells and lead to cytokine production, which might shape the immune response leading to allergic reactions. However, role of iNKTs in FA remains a less studied domain. A study of sphingomyelin from milk reported stimulation of iNKT towards a Th2 profile in milk-allergic children [180]. Another study showed increased secretion of IL-4 by a synthetic glycolipid ligand, a truncated sphingosine chain [181] derivative of α-galactosyl ceramide (most potent stimulator of iNKT known) known to induce secretion of IFNγ and IL-4 [182]. Endogenous lysosomal lipid antigen iGb3 which carries the terminal disaccharide, Gal-α-1,3-Gal, has been shown to be recognized by iNKTs in mice [183]. In conclusion, glycolipids depending upon the glycosyl and lipid composition, can induce cytokine secretion by iNKTs at a varying degree and drive immune response towards Th1 or Th2 polarization [184].

3.3. Immune response to 'α-Gal' in red meat allergic patients

In healthy individuals, anti-carbohydrate antibodies are naturally developed upon stimulation by gut microbes. These antibodies are usually of low affinity and largely IgM and IgG, earlier studies showed most of IgG towards carbohydrate of the isotype IgG2. However, more recent studies have shown approximately 50% of the anti-carbohydrate to be IgG2 [165, 166]. In a recent study, Zappe et al. detected >80% of IgG against the α-Gal epitope to be of the isotype IgG2 using a commercial intravenous IgG preparation from healthy individuals [185]. Interestingly, IgG subclasses in α-Gal-allergic patients show a distinct pattern compared to the healthy individuals as confirmed in previous studies. Increased levels of α-Gal IgG1 production with a background of IgG2 in allergic patients was reported by Rispens et al., which was further confirmed in two more studies, thereby showing a distinct IgG1 response in patients compared to a more general IgG2 response in healthy individuals [152, 154, 186]. A comparison of IgG subclasses to α-Gal in meat-allergic patients with that of IgG subclasses to a protein allergen in a protein-related FA, distinct specific IgG patterns are seen. Fish allergic individuals had higher IgG4 towards fish allergen and apple-allergic patients had IgG4 to Mal d 1, whereas no IgG4 response to α-Gal was detected in these studies [186, 187]. Kollmann et al. (2016) reports an increase in α-Gal IgG1 and IgG3 in allergic patients [187]. Thereby showing an increase in pro-inflammatory IgG (IgG1/IgG3) response in α-Gal allergic patients with a background increase of IgG2, however little or no increase in IgG4 was seen, thus differentiating IgG response against carbohydrates from that against protein allergens.

IgE response in individuals

Pre-existing IgG producing plasma cells can produce IgE upon direct or sequential class switching, either by direct class switching of IgM producing B cells which result in low affinity IgE antibodies or by sequential class switching of mature B cells which result in high-affinity IgE antibodies [188]. In humans, IgE production is mainly related to IgG1 and IgG4 producing B-cells in the presence of IL4/IL13 signalling via IL-4Rα and STAT6. Mature B cells stimulated with IL4 in the presence of anti-CD40 antibody produce IgG1 before class switching to IgE whereas immature transitional B cells preferentially directly class switch to IgE [189].

IV. Carbohydrate – a novel food allergen

Role of carbohydrates in allergy have been considered to be of low significance due to their weak immunogenic potential, and not surprisingly, very few carbohydrate allergens have been recognized in the allergen community. So far, α-Gal is the only globally recognized carbohydrate known to cause an allergic reaction, few other carbohydrate allergens have been reported but their occurrence is more localized. Another class of carbohydrates known in the field of allergy are cross-reactive carbohydrate determinants (CCDs). As the name suggests CCDs are responsible for high cross-reactivity, IgE directed against CCDs are of low clinical relevance but contribute to a high false-positive result in clinical settings, and hence CCDs have emerged as a major culprit in false-positive clinical test results.

4.1. Cross-reactive carbohydrate determinants

The term cross-reactive carbohydrate determinants (CCD) was coined by Aalberse et al. to describe carbohydrates present in various foods and insect venom that bound non-specifically to the IgE in patient serum. The binding was abolished by treatment of extracts with periodate thereby confirming presence of anti-CCD IgE. Certain carbohydrates are absent in humans and hence possess a capability to induce an immunogenic response; glycans from plants, insects, helminths etc. exhibit linkages such as α-1,3-fucose, α-1,6-fucose, and xylose etc. which are non-human. In addition, due to structural similarity with helminths certain glycans can induce IgE production and hence these carbohydrates are termed as cross-reactive carbohydrate determinants. CCDs are widely present in plants and insects, and most reports of anti-CCD IgE induction are against grass-pollen and hymenoptera stings [190]. Anti-CCD IgE are a recognized source of high cross-reactivity and false positive IgE-results in allergic patients, but they are largely of no clinical significance. Many studies have confirmed presence of anti-CCD IgE in asymptomatic patients, the lack of clinical reactivity is often attributed to low or no biological activity of the glycan molecules. A reported prevalence rate of 27-30% has been observed among patients with grass pollen/insect venom allergy [191].

Structure of CCDs: A typical glycan core structure is characterized by the presence of two N-acetyl-glucosamine and one mannose. Different glycan have different branching patterns, most studied ones are the CCD motifs MMXF (e.g. HRP), MUXF (such as in bromelain), and the MMFF (commonly found in insect allergens), see figure 8. Both MMXF and MUXF contain core-α-1,3-fucose and β-1,2-xylose, but different terminal mannose residue which forms an antenna-like structure in MMXF. MMFF on the other hand exhibits similar overall arrangement of sugars as MMXF but, instead of being xylosylated, it displays two fucose residues. Structural characterization showed common N-glycans (α-1,3-fucose) between plants and insect venom such as honeybee venom phospholipase A_2 [47, 190]. Different groups exhibit different structural features on their N-linked glycans where initial steps are conserved across all phyla with divergence starting at fungi [192] and second big divergence occurring at insects/plants/parasites, which have a different arrangement compared to the higher animals [193]. All plant species except moss have similar N-glycan arrangement, particular characteristic includes presence of core α-1,3-fucose, xylosylation of β-mannosyl and termination of glycans with mannose [190].

Prevalence: The prevalence of anti-CCD IgE in general population is not very well defined. In a cross-sectional study of schoolchildren (n=1328) from Ghana authors (Amoah et al.) observed peanut sIgE (≥0.35 kU/L) sensitization in 17.5% of the study population. However, most children had a negative skin prick test result and only 21 showed symptoms upon peanut ingestion pointing towards a large

number of false-positives. A strong correlation between peanut sIgE and anti-CCD IgE was detected among children indicating high cross-reactivity of CCD [194].

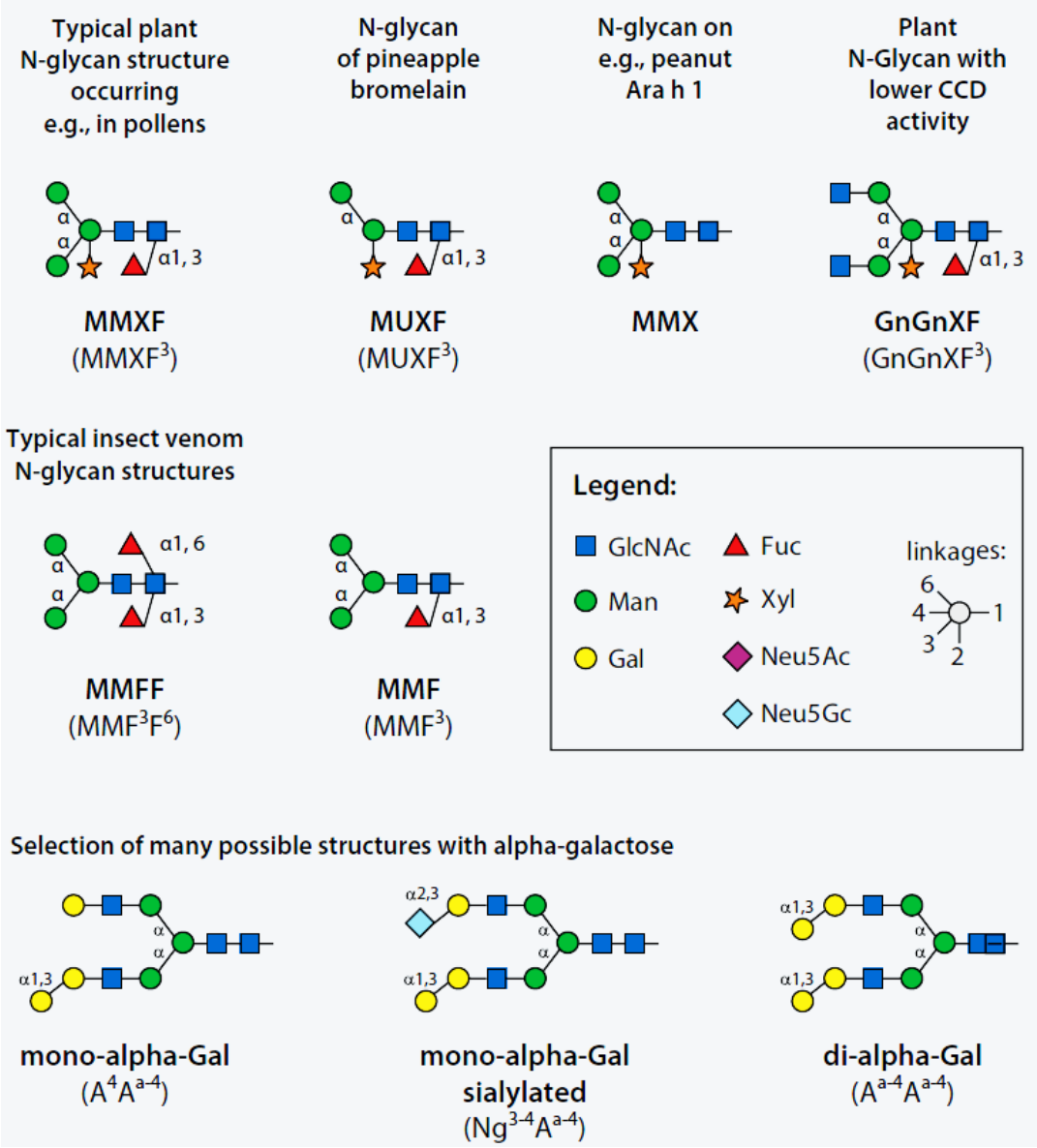

Figure 8. **Glycan structures occurring in plants, insects and CCDs with alpha-galactose**: top. examples of complex N-glycan structures found in plants, exhibiting β-1,2-xylosylation and α-1,3-core-fucosylation, middle. N-glycan structures commonly found in insect glycoproteins, with core α-1,3- and α-1,6-fucosylation, bottom. a selection of varied α-Gal modifications are shown (Fuc – fucose, Gal – galactose, Man – mannose, Xyl – xylose, GlcNAc – N-acetylglucosamine, Neu5Ac – N-acetylneuraminic acid, Neu5Gc – N-glycolyl-neuraminic acid). Reproduced from Altmann et al. [195]

4.2. Anti-CCDs in patients

Allergic patients especially those with pollen and insect venom allergy report higher prevalence of anti-CCD IgE [50]. In a study on 1831 subjects with suspected respiratory allergy an overall prevalence of anti-CCD IgE against plant N-glycan was 23%. Non-allergic individuals had 5% prevalence, non-pollen allergic 10%, and pollen-allergic individuals showed a 31% prevalence rate. Furthermore, a prevalence rate of 71% was observed in subjects with multiple pollen sensitization [196]. In a previous study, anti-

CCD IgE was detected in 30% of the insect venom allergic patients [197] whereas 10-50% of food allergic patients were positive for anti-CCD-IgE. In a study on sera from 6,220 allergic patients 22% were found to be positive [198]. In spite of the high diagnostic occurrence, clinical relevance of CCDs is low due to their inability in inducing clinical symptoms. However, the low clinical relevance isn't due to low binding affinity towards IgE but a high affinity of IgG blocking antibodies that inhibit IgE-binding (JIN et al.). An increased tolerance towards CCDs in highly exposed individuals such as in case of beekeepers can decrease their clinical relevance. However, an overall protective/aggravating role of anti-CCD IgE in allergic patients is not yet clear.

4.3. Clinically relevant carbohydrates

Cases of clinically relevant carbohydrate allergen are quite rare and so far only few have been identified – most important one being the globally recognized red meat allergy, two other forms are identified in the Asian subcontinent, bird's nest allergy and GOS allergy. Cases of bird's nest allergy are common in Singapore and the allergenic epitope, a 66-kDa glycoprotein, is thought to be a part of the carbohydrate derived from the nest-cementing substance. In another report on allergy to rice, CCD from rice was found to significantly contribute to the IgE binding, the structure is yet unresolved [143, 191].

A second major carbohydrate causing allergic reactions is galacto-oligosaccharide (GOS). GOS are prebiotics used as a supplement in commercial products like cow's milk formula. They are comprised of a mixture of glucose and galactose in oligomeric forms at linkages β-1,3, β-1,4 and β-1,6; they are naturally present in human and animal milk and can be enzymatically produced by treatment of lactose with bacterial-derived β-galactosidase [191]. Allergic reactions to GOS have been chiefly reported in Southeast Asia, the first case was reported in oyster farm workers in Japan with sea squirt allergy [199]. The workers developed immediate-hypersensitivity reactions to a lactic acid beverage and tested positive to GOS present in the consumed beverage, cross-reactivity between sea squirt antigen and GOS suggests origin of sensitizing molecule from sea squirts. Additional cases of GOS allergy were reported in children who were fed cow's milk formula supplemented with GOS [200]. Patients react to the complex GOS structures with 3 or more sugar units, notably not all GOS are allergenic, and the primary sensitizer remain undiscovered [143].

V. A vector-borne allergy

In the history of allergies, red meat allergy is the first to be characterized as a vector-borne allergy. In a usual scenario, allergic sensitization and effector route are the same. One anomaly observed in this context is the dual-allergen exposure, which states involvement of a different sensitization route than the one involved in effector cell activation. Red meat allergy is an addition to this category and affects the skin-gut axis i.e. sensitization via skin and allergic response via oral route. Since, its discovery various tick species have been identified as a sensitizing source; however, many questions remain unanswered.

5.1. The tick bite connection

Due to the onset of allergic response through oral encounter of α-Gal, the connection to tick bite was initially not apparent. As described previously in the section 2.2, a clear connection could be made after the discovery of α-Gal epitope in the context of cetuximab-mediated hypersensitivity [88]. By this time, cases of delayed allergic response to red meat were being reported in the southeastern region of US, detection of anti-α-Gal IgE in the sera of these allergic patients prompted further investigation. By then, Van Nunen and colleagues had already proposed the connection between red meat allergy and tick bites [90, 91]. Exploratory studies in this context focused on monitoring specific IgE response in patients bitten by ticks; additionally, correlation between titers of α-Gal sIgE and IgE to tick extract were analyzed to confirm the association [94]. Following observations in patients, association between tick bite and α-Gal allergy has been further well proved in knockout mouse models. Various tick species all over the world have been reported to sensitize including *Ixodes ricinus* (Europe), *Amblyomma sculptum* (Brazil), *Ixodes scapularis* (USA), *Amblyomma cajennense* (Costa Rica, Panama and Ivory Coast), *Ixodes holocyclus* (Australia), *Haemaphysalis longicornis* (Korea, Japan) etc. [55, 97].

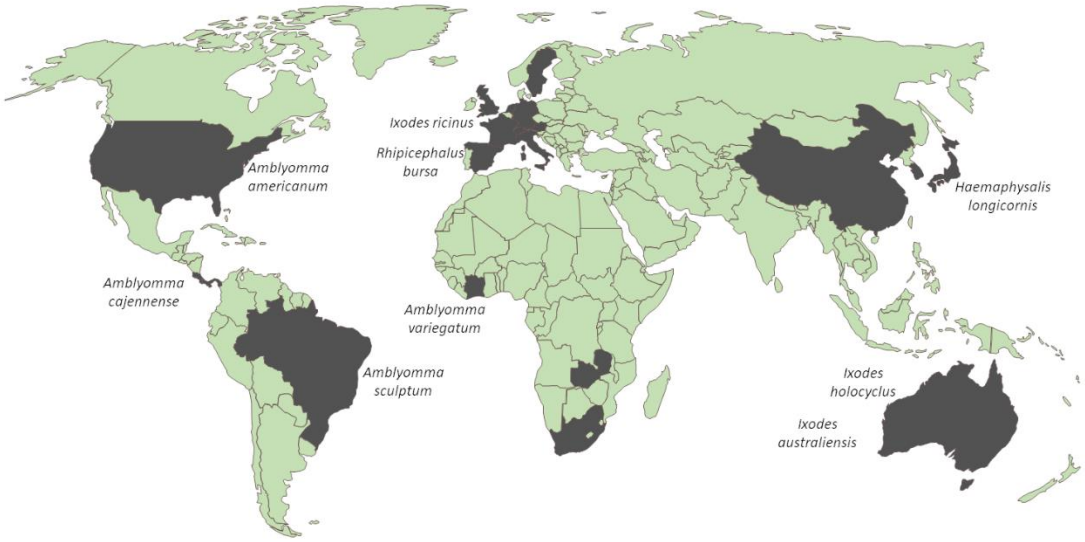

Figure 9. Numerous tick species have been reported to sensitize all over the world. Most important ones based upon frequency of the reports are – *Amblyomma americanum* (US), *Ixodes ricinus* (Europe), *Rhipicephalus bursa* (Spain) and *Ixodes holocyclus* (Australia). *Haemaphysalis longicornis* is reported in Asian countries (Japan, South Korea) and *Amblyomma sculptum* in Brazil. Reports on several other species have emerged which are more localized [55, 96-98]

5.2. Tick bites and sensitization

In recent years, several studies have successfully demonstrated production of α-Gal-specific IgE in α-1,3-galactosyltransferase knockout (α-1,3-GT-KO) mouse with either subcutaneous administration of the tick saliva/salivary gland extract or by direct feeding . In one of the first studies, Arajuho et al. reported production of anti-α-Gal IgE in α-1,3-GT KO mice upon subcutaneous injection of tick saliva as well as by direct feeding by the locally abundant tick *Amblyomma sculptum* [201], clearly demonstrating the tick-bite connection. Additionally, a Th2 polarizing role was observed by Chandrasekhar et al. (2019) when using larvae extract from lone star ticks (*Amblyomma americanum*) to immunize wildtype (with a-1,3-galactosyltransferase activity) as well as α-1,3-GT KO mice [202], which resulted in production of tick-specific IgE. Moreover, a clinical response could be established showing α-Gal sIgE production and allergic responses to pork fat and pork kidney in sensitized knockout mice [203]. Not only have these studies shown a direct role of tick bite in sensitization and in instigating symptomatic α-Gal allergy, but additional observations have further demonstrated the development of IgG response against α-Gal. Specific IgG response has been observed upon injection of α-Gal (Galα1-3Galβ1-4GlcNAc) conjugated to human serum albumin and mouse serum albumin [204]. In an interesting study on dogs (which naturally possess α-Gal), Hodzic et al. (2019) detected α-Gal-specific IgG, IgM, and IgE in sera and report production of α-Gal-specific IgM upon experimental infestation with the *Ixodes ricinus* ticks [205]. All these studies have clearly demonstrated a strong connection of tick bites to sensitization towards α-Gal. Next interesting question is source of α-Gal in ticks and the probable sensitizing mechanism.

5.3. α-Gal occurrence in ticks

Hamsten et al. first detected α-Gal in the gastrointestinal tract of *Ixodes ricinus* using patient sera and a polyclonal mouse anti-α-Gal antibody [206]; in a second study Fischer et al. (2020) detected α-Gal in the salivary gland, hemolymph and midgut of fed and unfed female adult *Ixodes ricinus* [207]. α-Gal containing proteins have been detected in the salivary gland of *Amblyomma americanum*, *Ixodes scapularis*, *Amblyomma sculptum*, *Haemaphysalis longicornis, Rhipicephalus bursa* and *Hyalomma marginatum* [201, 202, 204, 208-210]. However, the source of these α-Gal-carrying molecules in ticks remain elusive, especially due to the absence of α-1,3GT gene. Two studies have analyzed glycoforms in ticks: the first by Crispell et al. (2019) in which the authors detected glycoforms of α-Gal by N-glycan profiling of saliva from partially fed *Amblyomma americanum* and *Ixodes scapularis,* and in unfed salivary gland from *Ixodes scapularis* [209]. The second study by Park et al. (2020) characterized N-glycan profile of salivary glands of male and female *Amblyomma americanum* and detected an increase in α-Gal epitopes and cross-reactive carbohydrates over time after onset of feeding [208]. These studies further confirm the presence of α-Gal and its relationship with tick feeding. However, the glycans must be removed from carrier protein before analyzing them, and this makes it difficult to trace back the source of origin. Of note, mammalian proteins Vitellogenins and α-2-macroglobulin were identified as α-Gal containing proteins present in *Ixodes ricinus* showing host-originating molecules [211]. In conclusion, although several studies have identified sensitizing molecules in tick, the origin of these molecules is still a question of debate.

Prevailing theories on occurrence of α-Gal include – metabolic incorporation of host glycans into ticks, presence of residual glycan-carrying-molecules from previous blood meal, α-Gal contribution by tick microbiome or endogenous production by ticks [212]. Cabezas-Cruz et al. investigated the role of β-1,4-galactosyltransferase (β-1,4-GT) in heterologous expression of α-Gal in *Ixodes scapularis* and report assisted production of α-Gal [213]. However, a second study showed no reduction in α-Gal levels upon silencing of the β-1,4-GT but interestingly report a decrease in galactose metabolism and

transport in *Amblyomma americanum* upon silencing of α-D-galactosidase gene [210]. On the other hand, there is yet a lack of experimental evidence on the role of tick microbiome in allergic sensitization, thus leaving the question of the source of these molecules in ticks still unanswered.

5.4. α-Gal expression in ticks – the galactosyltransferase family

Galactosyltransferases belong to a family of glycosyltransferases that catalyzes transfer of galactose residue via α and β linkage at 1-2, 1-3, 1-4 (α-linkage) and 1-3, 1-4 (β-linkage) positions onto different acceptor substrates. α1,3-GALTs are responsible for expression of α-Gal epitope (galatose-α-1,3-galactose) onto glycoproteins and glycolipids. They belong to the GT6 family of glycosyltransferases that also includes enzymes such as iGb3 synthase, ABO blood groups (see detailed description on GT6 in section I). Previously, 57 GALT genes were identified in the tick genome, by searching *Ixodes scapularis* genome using GALTs from model organisms. The identified genes belonged to the families GT7 (β1,4-galactosyltransferases), GT31 (β1,3-galactosyltransferases), and GT32 (α1,4-galactosyltransferases). Most interestingly, the authors couldn't identify any member from GT6 (α1,3-galactosyltransferases) family which is responsible for galactose transfer at α-linkage and this includes GGTA1 gene along with ABO and iGB3S genes [213]. Thus, establishing absence of α-1,3-GT activity in ticks. However, some α-Gal production in *Ixodes scapularis* was shown and therefore hypothesizing heterologous expression, which could be attributed to the tick genes b4galt7, a4galt-1, and a4galt-2. However, more studies are needed to confirm heterologous gene expression as another study involving silencing of b4galt7 didn't see any decrease in the α-Gal levels [210], of interest is then to investigate the retention of host blood meal in ticks.

In a second hypothesis, a major source of allergen molecules could be the residual host blood from previous meal, which can be regurgitated into the next host upon subsequent feeding. Ticks are quite robust vectors feeding 3-4 times in their life span, which can range from 0.5-3 years depending upon the species and feeding opportunity [214]. They have evolved to feed on huge amounts of blood, and are able to store it for a long duration in order to survive lengthy periods of inactivity. Ticks can therefore retain host blood residues and carry various pathogens, which can easily enter into the host and drive immune reactions leading to infections. A previous study has identified residual mammalian host blood components in *Ixodes scapularis* and *Amblyomma americanum* when fed on rabbit, sheep and mouse; and a second study detected presence of host blood proteins in *Ixodes scapularis* after moulting, pointing towards a long retention of host blood in these tick species [215, 216]. Therefore, there is a possibility of α-Gal-carrying molecule from residual mammalian host blood are acting as a sensitizing source.

5.5. Sensitization *via* tick bite – plausible mechanism?

Although several studies have shown sensitization to α-Gal upon tick bite or subcutaneous administration of tick saliva/salivary gland extract in α-1,3-GT-KO mouse model, the exact sensitization mechanism remains undiscovered. Tick saliva is known to be Th2 polarizing, and several Th2 favouring molecules such as prostaglandins, phospholipases, adenosine, lipocalins etc., have been identified. These are known to inhibit IL2 and TNFα production from DCs and promote IL10 secretion, thereby, suppressing Th1 response to enable longer attachment and feeding on the host [217-219]. This microenvironment drives dendritic cells, mast cells, and ILC2 to promote Th2 cell and T follicular helper-2 (Tfh2) cell development, which results in the generation of IgE. Additionally, damaged epithelial cells secrete cytokines IL25, IL33, and TSLP which serve as early warning signal to the hematopoietic immune cells. Epithelial cells can in addition recognize pathogen-associated molecular

patterns (PAMPs) and danger-associated molecular patterns (DAMPs) by specific pathogen recognizing receptors (PRRs) [220, 221]. Most importantly, due to an evolutionary role in protection against pathogens in humans it is possible that α-Gal itself can act as PAMPs. Additionally lectins are known to bind to carbohydrate epitope and galectin-3 has been shown to recognize α-Gal. There is evidence of promotion of Th2 response upon recognition of carbohydrate-associated allergen by lectin [222]. Previously, a role of galectin-3 in allergy was demonstrated in murine model of airway remodelling and galectin-3 has been shown to recognize α-Gal [223].

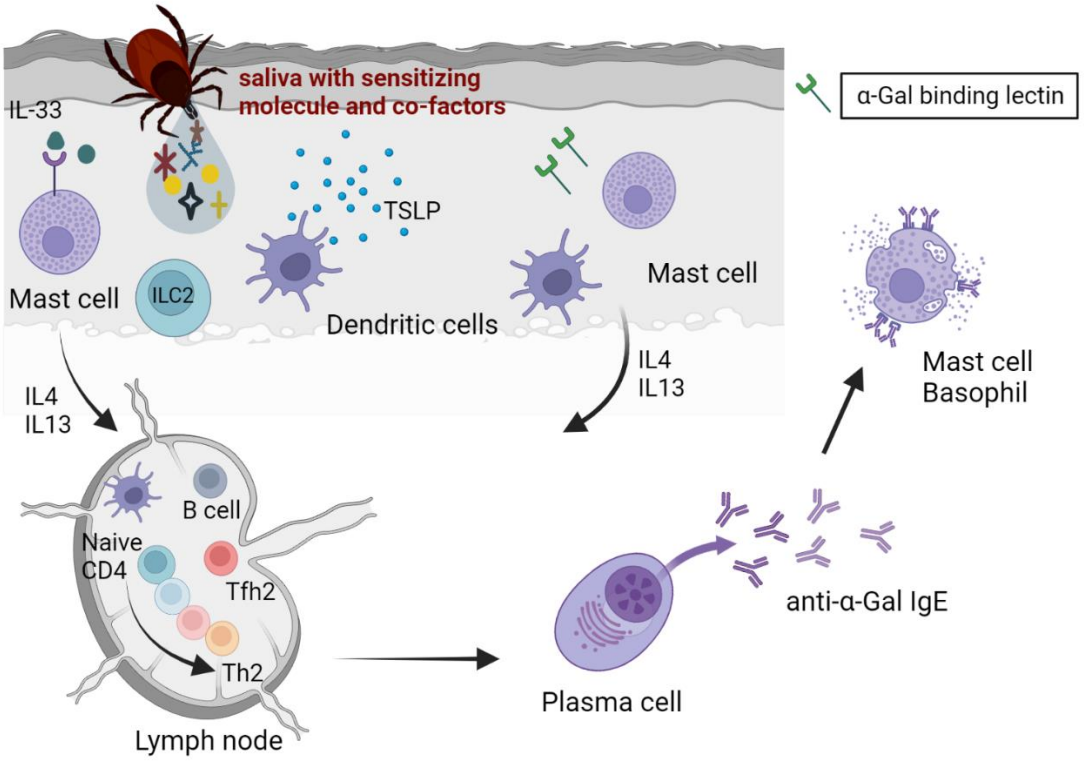

Figure 10. **Plausible α-Gal sensitization mechanism:** Bites from a hard tick can introduce α-Gal-carrying molecules (glycoproteins or glycolipids) into the skin while feeding. Cytokines released by epithelial cells at the site of lesion such as IL-25, IL-33, TSLP promote Th2/T follicular helper cell differentiation with the help of immune cells (ILC2, mast cells, and dendritic cells) which leads to B-cell class switching and production of IgE. Moreover, α-Gal can be recognized as PAMP (pathogen-associated molecular pattern) by lectin receptors that recognize and bind to carbohydrates, thus resulting in a direct stimulation of IgE production by the B-cells. Created in Biorender, adapted from Wilson et al. [221]

Additionally, involvement of a carbohydrate epitope is somewhat unique feature presented in this case which opens up two possibilities – firstly, B cells could be directly class-switched into producing IgE when triggered by the innate immune response. As carbohydrates are normally considered T-cell-independent another possibility could be class switching of pre-existing memory B-cells which could lead to IgE secretion even when T-cell help is absent. Secondly, as discussed in section III on carbohydrate immune response, carbohydrates can be independently recognized by T-cells when presented by MHCII molecules bound to a peptide/protein. Moreover, α-Gal on a glycolipid could be presented to iNKT-cells *via* CD1d receptors. Thus, a strong possibility of immune cell activation exists when α-Gal is present in conjugation with a carrier protein/lipid molecule.

5.6. *Ixodes ricinus* – major tick in Europe

Ixodes ricinus is the major causative vector of α-Gal allergy in Europe with cases reported in Sweden, Germany, Italy, France, Switzerland, Spain, and Norway [97]. Recent work in our department by Cramaro et al. presented a multiple-omics study on *Ixodes ricinus*, providing a large dataset of annotated transcripts and proteins of the naïve midgut [224]. This dataset was found to be devoid of α-1,3-GT activity. Searches across tick databases for sequences homologous to mammalian α-1,3-GT were negative. The Ixodes ticks are obligatory hematophagous ectoparasites that feed on vertebrates, feeding once at each developmental stage of their life cycle and have a robust survival mechanism, with a lifespan ranging from 6 months to several years [214]. They can consume up to 200-600 times their body weight of blood and go on prolonged starvation periods extending many months [225].

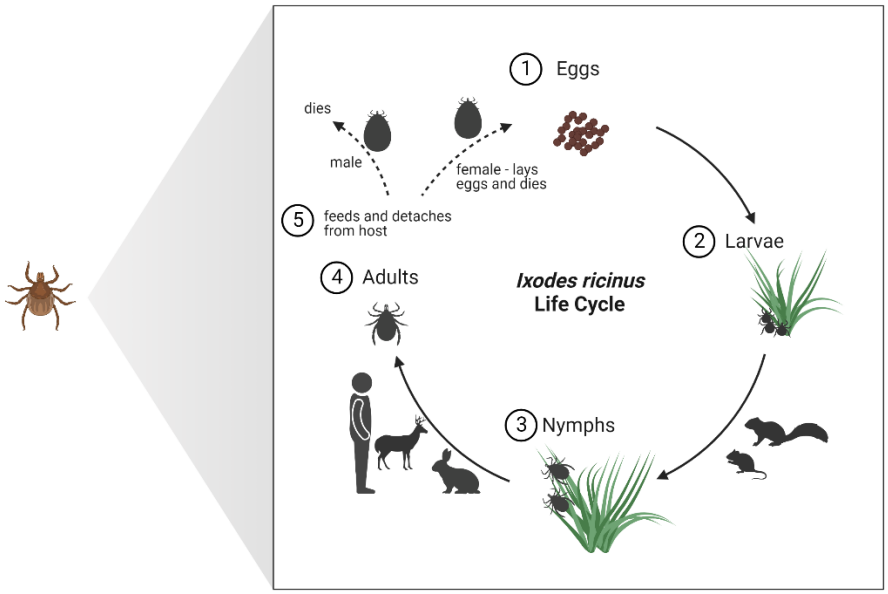

Figure 11. **Life cycle of *Ixodes ricinus***: *Ixodes ricinus* undergoes a three-host life cycle – larvae is usually found on ground close to the base of grass and feeds on small animals, upon moulting into nymphs they can climb and reside on top of grass, shrubs etc. and wait for the next host, they feed normally on larger animals. Adult ticks mate on top of large animals, the male feeds very little, detaches from host, and dies after mating, whereas female ticks feed and fall off the host, lays eggs and die. The entire life cycle can last from few months to several years depending upon the environmental condition and feeding opportunities.

Life-cycle: Ticks prefer a warm and humid environment. *Ixodes ricinus* shows a bimodal activity period with peak activity during early spring and late autumn. They operate on a three-host life cycle, feeding once per developmental stage from larvae to adult. Larvae are generally found close to the ground, in more humid environments and feed on small animals; after feeding they drop-off of the host and moult into nymphs. Nymphs can climb on top of grass or shrubs and often wait there for a host. They therefore normally feed on larger animals, and in Europe and America, nymphs are considered responsible for biting humans [214, 225]. Adult male and female ticks mate on top of the host and male ticks drop off and die, normally, not feeding or feeding very little, whereas female ticks feed, drop-off the host to lay eggs and then die. *Ixodes ricinus* can take from 6 months to several years to complete their life cycle depending upon host availability and their questing behavior [214].

5.7. Source of α-Gal in *Ixodes ricinus*

α-Gal was first detected in the gastrointestinal tract of the *Ixodes ricinus* by Hamsten et al. by staining cryostat-cut sections of nymphs with a polyclonal anti-α-Gal antibody as well as with patient sera. A more detailed analysis was recently presented by Fischer et al., in which they examined spatial distribution of α-Gal in unfed and fed female adult ticks by histology, immunostaining, and electron microscopy. By using lectin (*Marasmius oreades* agglutinin) and α-Gal-specific mouse monoclonal (clone M86) they report detection of α-Gal in midgut, hemolymph, salivary gland and in the ovary of adult female ticks. However, it still remains unclear if the epitope is synthesized by ticks or present on the residual host blood molecules.

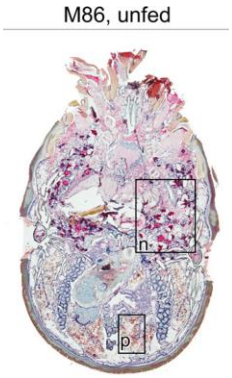

Figure 12. Coronal section of an unfed adult female *Ixodes ricinus* after starvation for five months – staining with M86 (red) antibody. n – shows salivary gland, and p – shows midgut; brownish-black appearance indicates presence of hematin particles in the midgut. Reproduced from Fischer et al. [207]

Unlike mammals where α-Gal epitopes are detected at varying concentration in all organs, authors could detect α-Gal in specific locations only. Moreover, in the lumen of fed ticks α-Gal was detected on fragmented blood cells indicating recognition of mammalian α-Gal. Additionally, authors could show a metabolic incorporation of mammalian α-Gal-containing molecules in the digestive cells, thus further conform digestion in the midgut epithelium. Detection of α-Gal in the hemolymph is interesting as previous studies have shown large quantities of storage protein in the hemolymph of adult ticks. These proteins can bind and store host-derived molecules [207]. In spite of several studies on the presence of α-Gal-carrying molecules in ticks, the source of origin of these molecules in ticks is still unclear. An understanding of host blood processing in ticks and retention over a span of time will help us further shed some light on the mechanism behind induction of α-Gal sensitization in humans upon feeding.

VI. Project Summary

IRGal *– "Targeting Key Factors Involved in* ***I****mmune* ***R****eactions mediated by Tick Bites to the Carbohydrate α-****Gal****"*

IRGal is a collaborative project among Luxembourg Institute of Health, University of Tübingen, Centre Hospitalier de Luxembourg, University of Hohenheim, and Technical University of Munich, running from 08/2018 – 07/2022. The project aimed at studying various aspects of red meat allergy, and is built upon previous work identifying α-Gal-carrying proteins from pork kidney followed by a successful demonstration of clinical relevance of the identified glycoproteins using basophil activation test and positive skin prick test in α-Gal-allergic patients [121]. The current project sought to extend the understanding by answering questions on the sensitizing molecule source, immune mechanisms in patients and plausible mechanisms behind the nature of delayed allergic response.

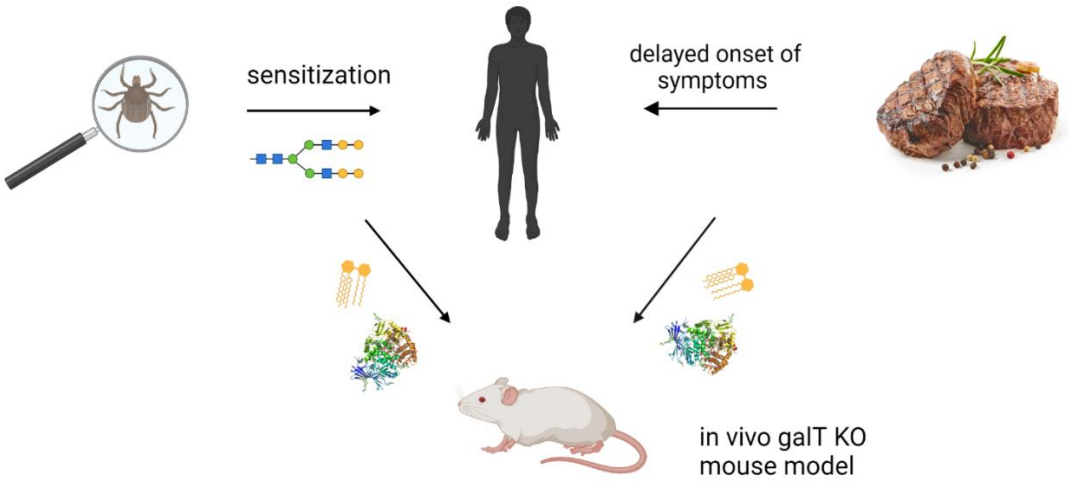

Figure 13. **Project IRGal**: this project focuses on the three aspects of α-Gal allergy – source of the sensitizing molecule, mechanisms behind delayed occurrence of the allergic reactions (molecular and immunological), and use of α-1,3-GalT KO mouse as a sensitization model. Collaborators at TUM conducted study on the mouse model.

PERSONAL CONTRIBUTION

As part of the PhD project, I have analyzed the presence of mouse blood in ticks in collaboration with the proteomics facility at LIH. Secondly, I have extracted and characterized glycolipids from various sources and compared their allergenicity to that of glycoproteins, in order to understand the mechanism behind the delayed allergic response, published article [142]. In addition, I have analyzed demographic data of a high-risk cohort and studied variation in α-Gal IgG subclasses and its correlation with tick bites. Lastly, I have made an attempt to generate mutant aminopeptidase N clones with varying epitope arrangement with the aim to use them in studying the role of spatial distribution in effector function mechanisms in case of a carbohydrate (α-Gal) allergen molecule. Figure 14 is a summary of contributions to the project IRGal.

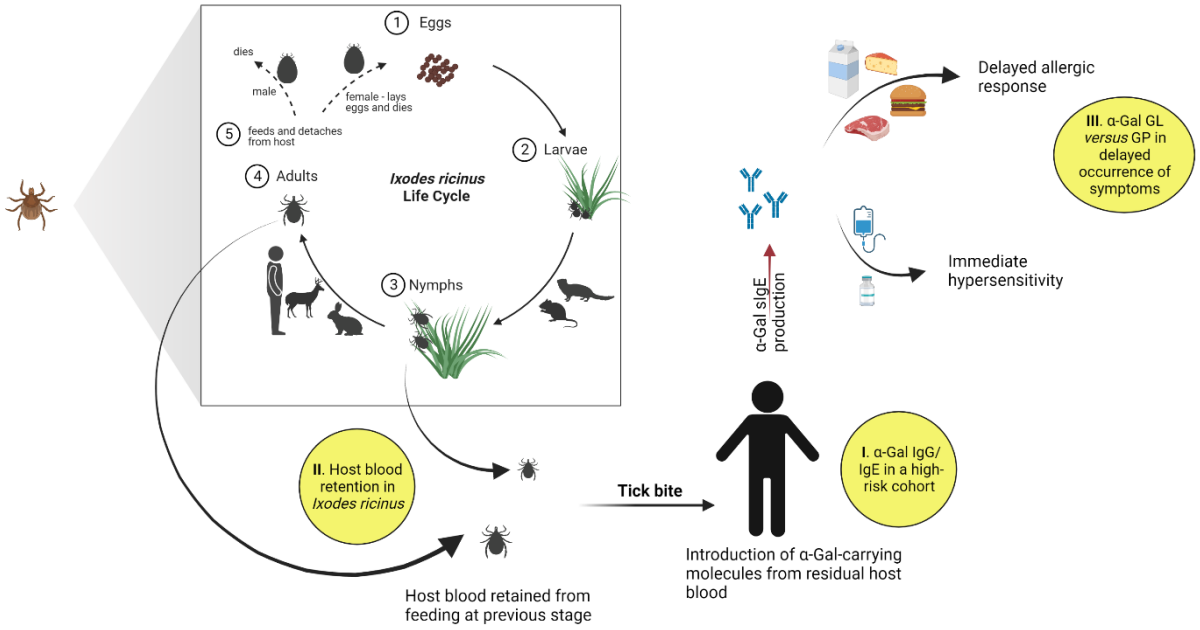

Figure 14. **PhD contribution overview**: In the context of this thesis three major studies were performed. I. α-Gal IgE prevalence in a high-risk cohort (Luxembourg Forestry Employees), II. analysis of *Ixodes ricinus* to detect presence of mouse blood after moulting and upon starvation, III. allergenic potential of glycolipids *versus* glycoproteins in the context of red meat allergy and possible role in the delayed occurrence of symptoms.

THESIS STRUCTURE

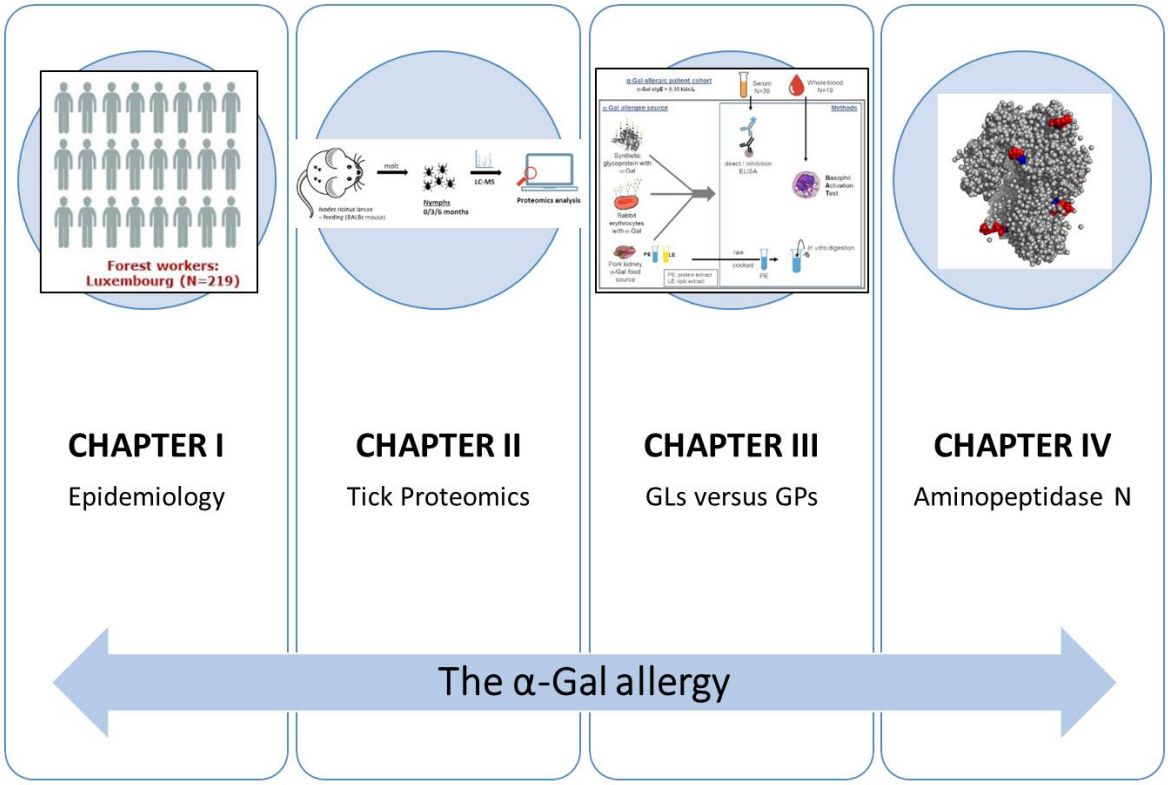

Figure 15. **Thesis outline**: chapter I and II are manuscripts (in preparation), chapter III is a published article and chapter IV is unpublished work.

Chapter I. Immune response (IgG/IgE) against α-Gal in a high-risk cohort of Luxembourgish forestry employees compared to allergic patients

Chapter II. Longitudinal study exploring retention of mouse blood protein in *Ixodes ricinus* nymphs to understand source of sensitizing molecule

Chapter III. Characterization and comparison of α-Gal-carrying glycoproteins and glycolipids as well as protein and lipids from natural food sources, to gain mechanistic insight into delayed occurrence of the allergic response

Chapter IV. To engineer aminopeptidase N molecules with α-Gal-glycosylation at specific positions to obtain model mutants. Second objective was to use these models to study the role of spatial arrangement of α-Gal in IgE-effector function

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SCOPE AND AIMS

Red meat allergy aka α-Gal allergy is a delayed allergic response occurring upon consumption of mammalian meat and by-products. Patients report eating meat without any problems for several years before developing the allergy. Although children can develop red meat allergy, it is more prevalent in adults. In addition to a delayed onset of reactions, immediate hypersensitivity is reported in case of contact with the allergen via intravenous route. Galactose-α-1,3-galactose (α-Gal) is the first highly allergenic carbohydrate that has been identified to cause allergy all across the world. In general, carbohydrates exhibit low immunogenicity and are not capable of inducing a strong immune response on their own. Although the α-Gal epitope is present in conjugation with both proteins and lipids, due to an overall accepted role of proteins in allergy, glycoproteins from mammalian food sources were first characterized. However, a unique feature of α-Gal allergy is the delayed occurrence of allergic symptoms upon ingestion of mammalian meat and an allergenic role of glycolipids has been proposed to explain these delayed responses.

A second important feature of the disease is that the development of specific IgE to α-Gal has been associated with tick bites belonging to various tick species, depending on the geographical region. In this tick-mediated allergy an intriguing factor is the absence of an α-1,3-GalT gene in ticks, a gene coding for an enzyme capable of α-Gal synthesis, which raises questions on the source and identity of the sensitizing molecule within ticks, immune responses to tick bites, and effect of increased exposure.

In this study, we sought to elucidate the origin of sensitization to α-Gal by investigating a cohort of individuals exposed to recurrent tick bites and by exploring the proteome of ticks in a longitudinal study. Furthermore, we analysed the allergenicity of glycoproteins and glycolipids in order to determine the food components responsible for the delayed onset of symptoms.

The aim of the **Chapter I** was to determine IgG profiles and the prevalence rate of sensitization to α-Gal in a high-risk cohort of forestry employees from Luxembourg.

The aim of **Chapter II** was to analyse the presence of host blood in *Ixodes ricinus* after moulting and upon prolonged starvation in order to support or reject the host blood transmission hypothesis.

The aim of the **Chapter III** was to investigate and compare the allergenicity of glycolipids and glycoproteins to understand their role in the allergic response. Moreover, we have analysed the stability of glycoproteins and compared extracts from different food sources. This chapter is in the form of a published article.

In **Chapter IV**, I have made an attempt to create mutant models with specified α-Gal glycosylation in order to study role of spatial distribution of α-Gal in IgE cross linking and effector cell activation.

CHAPTER I

CHAPTER I: Anti α-Gal antibody responses in a cohort of Luxembourgish forestry employees show distinct IgG and IgG subclass profiles

Neera Chakrapani,…., Christiane Hilger (2022). Anti α-Gal antibody responses in a cohort of Luxembourgish forestry employees show distinct IgG and IgG subclass profiles*. Manuscript in preparation*

Author contributions:

In this project, I have conceptualized the study together with CH and KS. I compiled and analysed the epidemiological data. KS has performed the experimental part of the study – total IgE detection and IgG subtyping. I have analyzed the data and prepared the manuscript with inputs from CH. KS has critically reviewed the data.

MANUSCRIPT

Anti α-Gal antibody responses in a cohort of Luxembourgish forestry employees show distinct IgG and IgG subclass profiles

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ABSTRACT

Background: Red meat allergy or α-Gal syndrome is characterized by the presence of specific IgE antibodies to the carbohydrate galactose-α-1,3-galactose (α-Gal) and a delayed onset of allergic symptoms after ingestion of mammalian meat. Sensitization to α-Gal has been associated with tick bites and individuals exposed to ticks have an elevated risk of sensitization.

Objectives: To analyse IgG and IgE antibody responses to α-Gal in an occupational cohort of forestry employees in Luxembourg.

Methods: Questionnaires and serum samples from a cohort of forestry employees from Luxembourg (N=219) were retrospectively analysed. α-Gal specific IgE were tested by ImmunoCAP, α-Gal specific IgG and IgG subclasses IgG_{1-4} were determined by ELISA. Sera from two groups of food-allergic patients, patients with red meat allergy (N=45) and fish-allergic patients (N=22), were also assessed for IgG antibody responses to α-Gal and cod extract.

Results: In forestry employees, 21% were sensitized to α-Gal (sIgE≥0.1 kU/L). There was no significant difference between sensitized and non-sensitized individuals in terms of years in occupation or number of experienced tick bites. Employees with an atopic background were more likely to have α-Gal sIgE (OR 5.7). Both sensitized and non-sensitized forestry employees exhibited high levels of α-Gal specific IgG, IgG1 and IgG2 when compared to fish-allergic patients, indicating a stimulation of the IgG response by recurrent tick bites. Fish-allergic patients had low IgG2 antibody levels to α-Gal, IgG1 were absent. A comparison of IgG levels to α-Gal in meat-allergic patients to IgG against cod extract in fish-allergic patients showed significantly higher levels of IgG and IgG_{1-3} in meat-allergic patients, but almost no IgG4, whereas fish-allergic patients had low, but significant IgG4 levels to fish.

Conclusion: Our study provides an evidence for a continuous stimulation of α-Gal related immune responses by repeated bites, translating into elevated levels of IgG1 and IgG2 antibodies directed against α-Gal. Significant levels of α-Gal specific IgG4 were not detectable in forestry employees, nor in meat-allergic patients, a feature that seems to be specific to the α-Gal allergy.

INTRODUCTION

Red meat allergy is a novel form of tick-borne disease characterized by a delayed onset of allergic reaction, usually within 2-6 hours, after consumption of mammalian meat and by-products (1). Van Nunen and colleagues first reported the connection with ticks when 25 patients in a tick-endemic region in Australia presented with a delayed allergic reaction to red meat and all except one re-called tick-bites and all described local reaction to the bites (2). These findings were further confirmed in case reports from the southeastern states of US, when cancer patients developed hypersensitivity reactions to the chimeric monoclonal mouse-human IgG1 antibody cetuximab which is directed to the epidermal growth factor receptor (3) and used in treatment of colorectal and squamous-cell carcinoma of the head and neck. Analysis of pretreatment sera of these patients confirmed the presence of anti-α-Gal specific IgE that recognized the terminal glycan epitope galactose-α-1,3-galactose present on the Fab fragment of cetuximab (4). Numerous cases of red meat allergy aka α-Gal allergy have been reported in Europe (5-13), and cases are emerging in Africa, South America, and Asia (14), making it a globally recognized allergy. Identified causative tick species are different on each continent and *Amblyomma americanum*, *Ixodes ricinus, Ixodes holocyclus,* and *Haemaphysalis longicornis* are the most frequently reported ones (14, 15). In Europe *Ixodes ricinus* is responsible for sensitization (14), and α-Gal has been detected in the midgut, hemolymph, salivary gland and ovary of the female adult ticks (16, 17).

All mammals naturally express α-Gal on glycoprotein and glycolipid structures, except humans, apes, and old-world monkeys due to a frame shift mutation occurring 20-28 million years ago which resulted in inactivation of the gene for α-1,3-galactosyltransferase (α-1,3-GT) thereby stopping α-Gal expression (1). Owing to this, humans produce anti-α-Gal antibodies upon antigenic stimulation, which is thought to provide an evolutionary advantage by playing a protective role against pathogens and bacteria (18). Early studies by Galili et al. report as much as 1% of the antibodies in humans as anti-α-Gal (19), however additional studies report lesser quantities (20, 21). Nonetheless, anti-α-Gal antibodies remain an abundant antibody in humans and are especially responsible for rejection of mammalian grafts (19). Studies have identified anti-α-Gal IgG, IgM, and IgA antibodies in human serum (22) and IgA in secretions such as saliva, milk, vaginal washings etc. (23).

As sIgE directed at α-Gal are strongly associated with tick bites, populations with a higher frequency of exposure, either due to geography or due to occupation, are also at a greater risk of being sensitized. High sensitization rates were reported in forestry workers from Germany (35%) and timber harvesters, and forestry and wildlife practitioners from Kentucky (40%) (24, 25). Moreover, pointing to a major role of living area and leisure activities, Villalta et al. confirmed an increased sensitization prevalence in rural populations and hunters (26). Most studies found a correlation of sIgE with the number of tick bites, outdoor activity and seniority (8, 24, 25, 27, 28), whereas increased risk due to polysensitization (29) and tick bite within 12 months of sampling (6) have been reported as well. Venturini et al. show an association of α-Gal sIgE positivity to number of tick bites per year (28). In populations with lower exposure to ticks, sensitization rates have been reported between 4% – 8.1% (27-29), whereas patients with insect venom allergy tend to have higher α-Gal sIgE prevalence (6).

α-Gal allergic patients possess higher levels of α-Gal specific IgG compared to healthy individuals (30-32), furthermore they are found to produce higher levels of α-Gal IgG1 (30) whereas in commercial pharmaceutical human immunoglobulin preparations α-Gal IgG2 was the dominant subclass (33, 34). This finding was confirmed in healthy blood donors (35). In the present study, we aimed at determining the α-Gal specific IgE prevalence rate among Luxembourgish forestry employees and the effect of various demographic factors on sensitization. In addition, as several studies showed increased levels of α-Gal IgG in α-Gal-allergic patients, we aimed at analyzing factors contributing to the rise in α-Gal

IgG and IgG subclass levels in a high-risk cohort subjected to frequent tick bites. Our study results confirm a direct role of tick bites in increased IgG levels to α-Gal. Furthermore, we identified distinct IgG subclass profiles when comparing IgG anti-α-Gal antibodies present in α-Gal-allergic patients to anti-cod antibodies in a control group of fish-allergic patients.

METHODS

Study population

Forestry Employees

Forestry employees were recruited in 2012 and 2013 in collaboration with the Nature and Forest Agency in Luxembourg in the frame of study on Lyme disease (MarLyBor, Ethical approval CNER N°201111/07). All participants gave their informed consent for participation in the study. Data on hours spent outside, year of occupation as forestry workers, number of tick bite the year before recruitment, date of last tick bite, gender and age were recorded on self-reported questionnaires. Only participants for whom serum samples were available were included in the present study (N=219). Serum samples were collected in May 2012 and April 2013.

Patients

Sera from symptomatic α-Gal–allergic patients previously recruited at the outpatient clinic of the National Immunology–Allergology Unit at the Centre Hospitalier de Luxembourg (N=35) and the Allergology Unit of the Department of Dermatology of Eberhard Karls University in Tubingen (N=10) were included in the study. Patients had a positive clinical history and were previously tested for total IgE and specific IgE levels to α-Gal (sIgE ≥0.35 kU_A/L) (ImmunoCAP; Thermo Fisher Scientific, Uppsala, Sweden). In addition, sera from fish-allergic patients (N=22) were used as food-allergic control population. The study was approved by the national committee for medical research ethics in Luxembourg (201307/04, 201605/03 and 201910/04) and by the ethics commission of the University Medical Faculty in Tubingen (158/2016BO1). Written informed consent was obtained from all study participants.

Specific and total IgE measurement

Specific IgE (sIgE) to α-Gal (galactose-α-1,3-galactose) was measured in ImmunoCAP (ThermoFischer) as per manufacturer's standard procedure. IgE against heated cod extract in fish-allergic patients was measured using an in-house ELISA (36), and total IgE was measured either via ImmunoCAP or using a total IgE detection kit (ThermoFischer Scientific) as described below.

For measurement of specific IgE to cod extract, plates were coated with cooked cod fish extract at 5 µg/ml in PBS. Cod fish extract was prepared from cod flesh, 0.5 gm per ml in single detergent lysis buffer (50 mM Tris-HCl, 150 mM NaCl, pH 8) with 1% Triton-X100, incubated for 10 minutes at 30 Hz with 5mm steel bead per 2ml tube in Retsch Mill and centrifugated for 30 minutes at 17.500 x g. The supernatant was heated for 10 minutes at 95°C, centrifuged for 15 minutes at 17.500 x g, and protein concentration measured using Bradford's assay.

Total IgE was measured with the human IgE ELISA kit from Invitrogen (ThermoFisher Scientific), following protocol provided by the manufacturer's. Briefly, 96-well ELISA plates (Maxisorp, Nunc, ThermoFisher Scientific) were coated overnight with the capture IgE antibody, followed by blocking and incubation with standards or sera diluted 1:10. Next, incubation with IgE detection antibody labeled with horseradish peroxidase (HRP) was followed by addition of the substrate tetramethylbenzidine (TMB, ThermoFisher Scientific). Reaction was stopped with stop solution (2N sulfuric acid) after 10 minutes and results were obtained on Spectramax iD3 multi-mode microplate

detection platform by subtraction of OD at 570nm from OD at 450nm. Finally, total IgE values were calculated by extrapolating OD values from the standard curve.

IgG subclass phenotyping

For α-Gal IgG subtyping, 384-well ELISA plates (high-binding, Greiner Bio-one, Germany) were coated with α-Gal coupled to human serum albumin (α-Gal HSA, Dextra Labs, Reading, UK) at 2 µg/ml in phosphate buffered saline (PBS) overnight at 4°C. Next day, plates are washed 3x with tris-buffered saline with 0.05% Tween-20 (TBST) and blocked with 1% HSA/TBST for 1 hour at room temperature. Thereafter, wells were washed and sera diluted in 0.5% HSA/TBST (1:50 for IgG determination and 1:20 for subclasses IgG1, IgG2, IgG3, and IgG4) was added and incubated at room temperature (RT) for 2 hours with horizontal shaking (300rpm). For secondary detection, plates were washed 3x in TBST and incubated with alkaline phosphatase (AP) labeled anti-IgG or anti-IgG1 diluted 1:2000, or anti-IgG2, anti-IgG3, and anti-IgG4 diluted 1:500 in 0.5% HSA/TBST for 2 hours at RT with horizontal shaking (300 rpm). For highly sensitive IgG4 phenotyping, coated and blocked plates were incubated overnight with sera diluted 1:10 and secondary detection performed the following day with anti-IgG4 labeled with biotin for 90 minutes, followed with streptavidin-AP for 30 minutes. Finally, plates were washed and developed with para-nitrophenyl phosphate solution (Sigma Aldrich) and optical density (OD) read at 405 nm on Spectramax 384Plus ELISA reader (Molecular Devices, UK). All antibodies were purchased from Southern-Biotech (UK).

Statistical analysis

IgE and IgG data were analyzed in GraphPad Prism v9 (GraphPad Software, La Jolla, California). Significance of demographic factors on sensitized *versus* non-sensitized groups was compared with Chi-square/Fischer's exact test. Differences in IgG subclass levels between two groups were determined by Mann-Whitney test, a p value of $p<0.05$ was considered statistically significant. Correlation between specific-IgE and total-IgE levels, IgG and IgG subclass levels within a cohort were analyzed with Spearman's correlation. Finally, significance of variation in IgG1 and IgG2 levels across groups were compared using Kruskal-Wallis with Dunn's multiple comparison test.

RESULTS

I. High α-Gal sIgE prevalence among Luxembourgish forest service employees

Forestry employees were recruited in 2012 and 2013, and data on age, gender, years in occupation, type of work, activity outside, history and number of tick bites were obtained through self-reported questionnaires. Serum was available from 219 participants and their demographic data are displayed in Table I. The vast majority of the participants spent more than 4 hours outside daily (86%) and reported tick bites (90%).

Table I. Demographic data of Luxembourgish forest employees

		Total cohort	Workers	Rangers	Occupation not defined	Sensitized (sIgE ≥0.1 kUA/L)	Non-sensitized (sIgE <0.1 kUA/L)	Chi square/Fischer's exact test (sensitized *vs* non-sensitized)
No.		219	167	42	10	46 (21%)	173 (79%)	--
Gender (male%)		211/219 (96%)	162/169 (96%)	39/42 (93%)	10/10	45/46 (98%)	166/173 (96%)	--
Age in yrs (median; range)		44.5 (19 – 63)	45 (19 – 63)	41 (23 – 60)	47 (24 – 51)	43 (19 – 63)	45 (19 – 63)	--
Activity outside (hrs/day)	≤4	29/211 (14%)	4/161	25/42	na	7/43 (16%)	22/168 (13%)	ns
	>4	182/211 (86%)	157/161	17/42	8/8	36/43 (84%)	146/168 (87%)	
Years in occupation	<10	59/205 (29%)	45/160	14/40	na	13/41 (32%)	46/164 (28%)	ns
	10 – 20	48/205 (23%)	38/160	9/40	1/5	11/41 (27%)	37/164 (23%)	
	>20	98/205 (48%)	77/160	17/40	4/5	17/41 (41%)	81/164 (49%)	
No. of tick bites/year	0	24/219 (11%)	18/167	5/42	1/10	4/46 (9%)	20/173 (12%)	ns
	1 – 5	111/219 (50%)	84/167	22/42	5/10	30/46 (65%)	81/173 (47%)	
	6 – 10	43/219 (20%)	33/167	8/42	2/10	5/46 (11%)	38/173 (22%)	
	>10	36/219 (16%)	27/167	7/42	2/10	7/46 (15%)	29/173 (17%)	
Last tick bite	Within 1 yr of collection	143/175 (82%)	119/139	31/33	3/3	33/36 (92%)	120/139 (86%)	ns
	>1 yr	22/175 (13%)	20/139	2/33	na	3/36 (8%)	19/139 (14%)	

The data are shown as absolute numbers (n/N). Total count (N) shows no. of employees that answered the questionnaire
yrs, years; hrs/day, no. of hours per day; na, not applicable; ns, not significant
*No. of tick bites/year reports no. of bites experienced annually

The prevalence of sIgE directed against α-Gal was determined by ImmunoCAP. Twenty-one percent (N=46) of the employees were sensitized (sIgE ≥0.1 kU_A/L) and of those, 57% (N=26) had sIgE levels of ≥0.35 kU_A/L (Fig. 1A). A significantly higher proportion of sensitized employees were atopic (78%) (total IgE ≥100 kU/L) compared to the non-sensitized employees (39%) (Fig. 1A). There was no significant difference in time spent outside between sensitized and non-sensitized employees (Table I). The prevalence of sIgE against α-Gal was not associated with outdoor activity of more than 4 hrs/day compared to ≤ 4 hrs/day (Table I, Fig. 1B). Levels of total IgE and α-Gal sIgE were correlated, Spearman r=0.6140 (Fig. 1B). Ninety-two percent of the sensitized and 86% of the non-sensitized employees reported tick bites within 1 year of sample collection (Table I); 65% of the sensitized participants reported between 1-5 tick bites whereas 47% of the non-sensitized individuals reported similar

number of tick bites (Fig. 1C). Overall, 19% of the employees reported a history of Lyme disease, whereas 13% of the sensitized employees had a history of Lyme disease (data not shown).

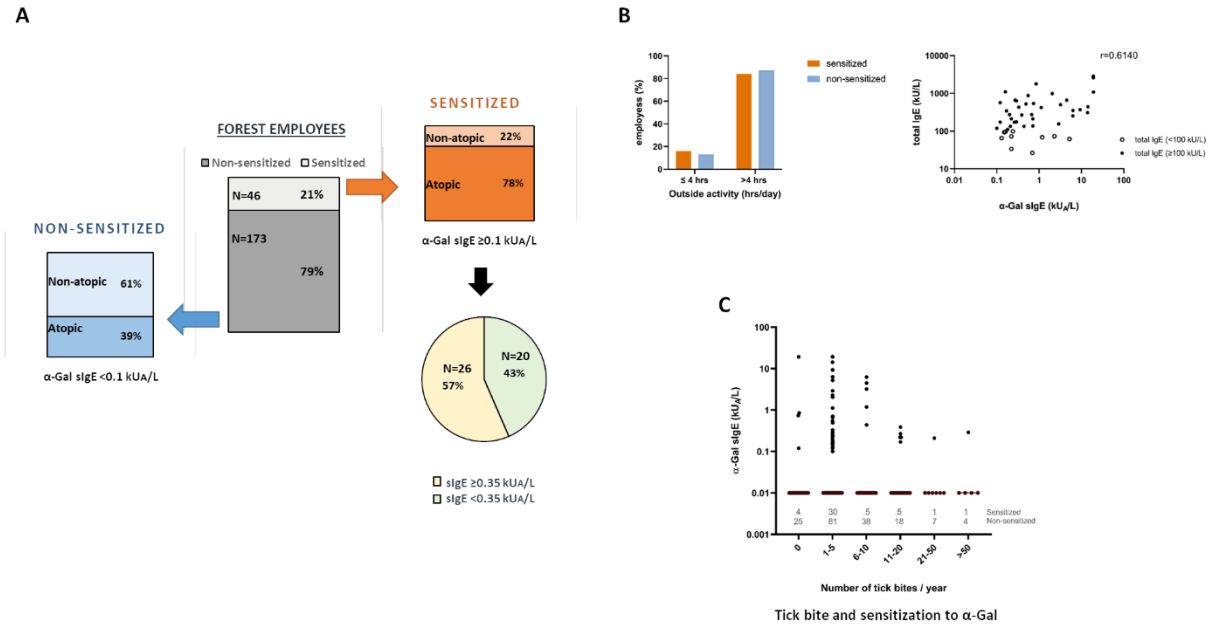


Fig 1. **Sensitization prevalence to α-Gal among Luxembourgish forestry employees: A.** Sensitization prevalence among forestry employees as detected by ImmunoCAP (α-Gal sIgE ≥0.1 kUA/L). Atopy (total IgE ≥100 kU/L) within sensitized and non-sensitized groups shown in percentage; highly significant difference in atopic status between sensitized and non-sensitized groups (Fischer's exact test, $p<0.0001$). **B.** Bar graph shows daily outdoor activity and percentage of sensitized and non-sensitized employees (left), chi square test – not significant (ns); a correlation plot of total IgE *versus* α-Gal sIgE, Spearman r=0.6140 (right). Open circle – non-atopic (total IgE <100 kU/L), closed circle – atopic (total IgE ≥100 kU/L). **C.** XY plot showing levels of α-Gal sIgE in sensitized employees *versus* no. of tick bites reported; the number of non-sensitized employees reporting tick bites is mentioned below the sensitized count (x-axis).

II. α-Gal IgG and subclass IgG levels are higher among sensitized forestry employees

Humans naturally produce anti-α-Gal IgG antibodies, but significantly higher IgG levels were described in meat-allergic patients than in controls (30-32, 35, 37). We therefore investigated anti-α-Gal IgG and IgG subclass levels in sensitized and non-sensitized forestry employees. Anti-α-Gal IgG and IgG1 levels were significantly higher in sensitized individuals (Fig 2A). In addition, a moderate, but significant increase in α-Gal IgG2 and α-Gal IgG3 levels was observed, whereas no significant difference in α-Gal IgG4 levels could be detected (Fig. 2A).

IgG1 and IgG2 are moderately correlated in non-sensitized employees, but there is no correlation between IgG1 and IgG2 levels among sensitized employees. IgG1 levels of sensitized employees cluster at high values whereas IgG2 values show highly variable levels (Fig. 2B). As the majority of measured IgG1 levels were close to assay saturation, all samples would need to be run at higher dilution in order to correctly assess any correlation (Fig. 2B, 3B, S1).

Furthermore, α-Gal sIgE levels among sensitized employees are weakly correlated with α-Gal IgG (Spearman r=0.3820) and IgG1 (Spearman r=0.4282) (Fig. S1). No significant difference was observed in IgG subclass levels in atopic (total IgE ≥100 kU/L) *versus* non-atopic individuals, in both sensitized and non-sensitized groups (Fig. S2).

As no significant difference in daily activities and exposure to tick bites was seen between sensitized *versus* non-sensitized employees, a difference in IgG levels likely points towards a difference in immune reactions to tick bites in both groups.

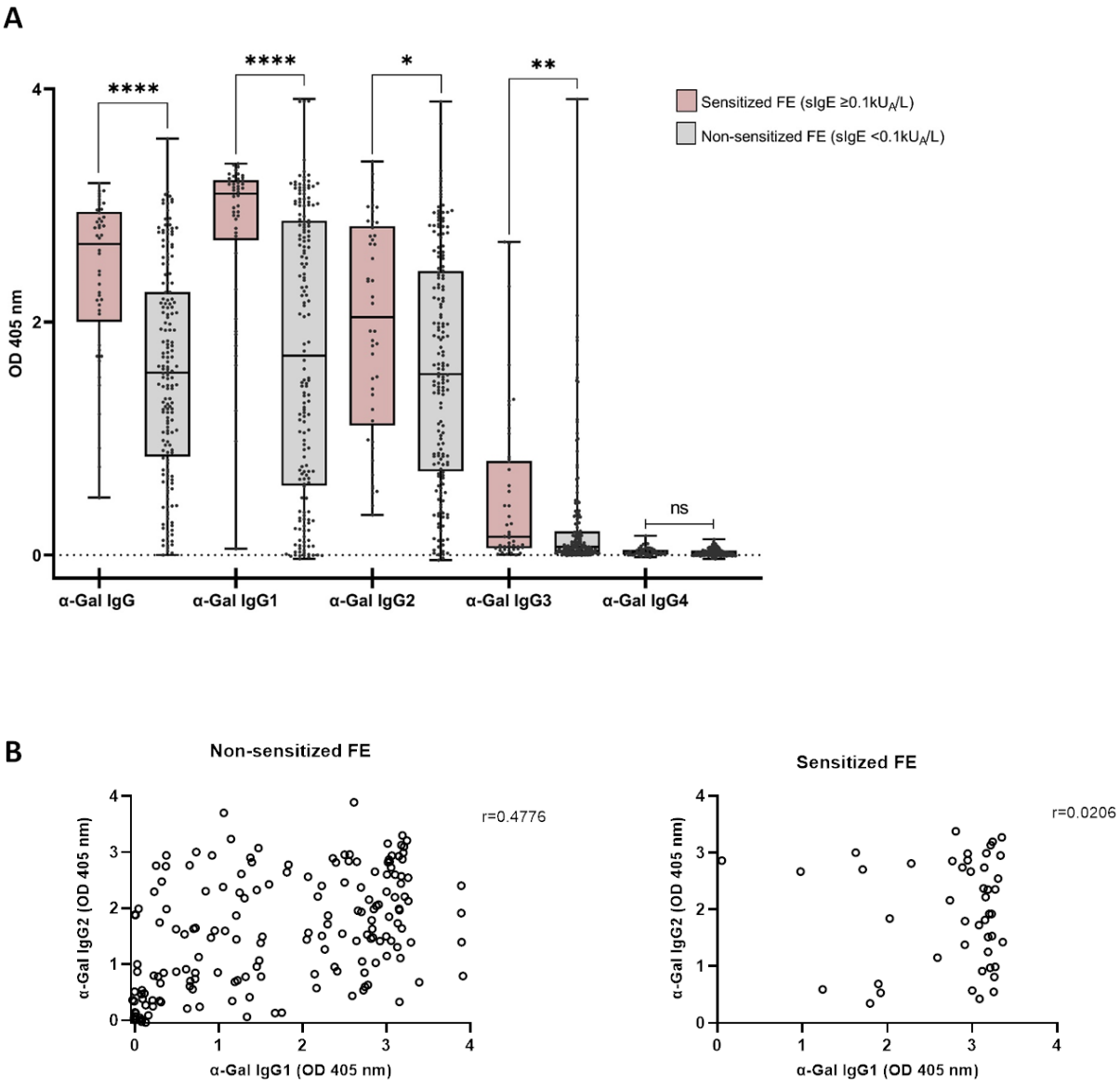


Fig 2. **Sensitized forestry employees produce significantly higher quantities of anti-α-Gal IgG: A.** Box plot showing levels of anti-α-Gal IgG/subclasses between sensitized (α-Gal sIgE ≥0.1 kUA/L) and non-sensitized groups (x-axis: IgG subclass, y-axis: optical density at 405 nm). Mann-Whitney comparison of differences in antibody levels between groups (* p<0.05, ** p<0.01, **** p<0.0001, ns: not significant). Whiskers extend to minimum and maximum values, median displayed as line within each box. **B.** Spearman correlation (r) between anti-α-Gal IgG1 and IgG2 subclass levels within non-sensitized (top; r=0.4776, p<0.0001) and sensitized (bottom; r=0.0206, p value not significant) groups.

III. α-Gal-allergic patients have higher levels of IgG anti-α-Gal antibodies than sensitized forestry employees

Having found highly significant differences in levels of IgG and IgG subclasses between sensitized and non-sensitized individuals, we also analyzed the anti-α-Gal IgG patterns among α-Gal-allergic patients (Table II) and compared them to the IgG profiles of sensitized forestry employees. α-Gal-allergic patients had significantly higher anti-α-Gal IgG1 and IgG2 levels than the sensitized forestry employees (Fig. 3A). However, correlations between IgG1 and IgG2 were either non-existent (sensitized group) or moderate (α-Gal-allergic) (Fig. 2B and 3B). sIgE levels were higher in allergic patients, and IgG2 were moderately correlated with sIgE levels (Fig. 3B and 3C), whereas IgG1 were not (data not shown).

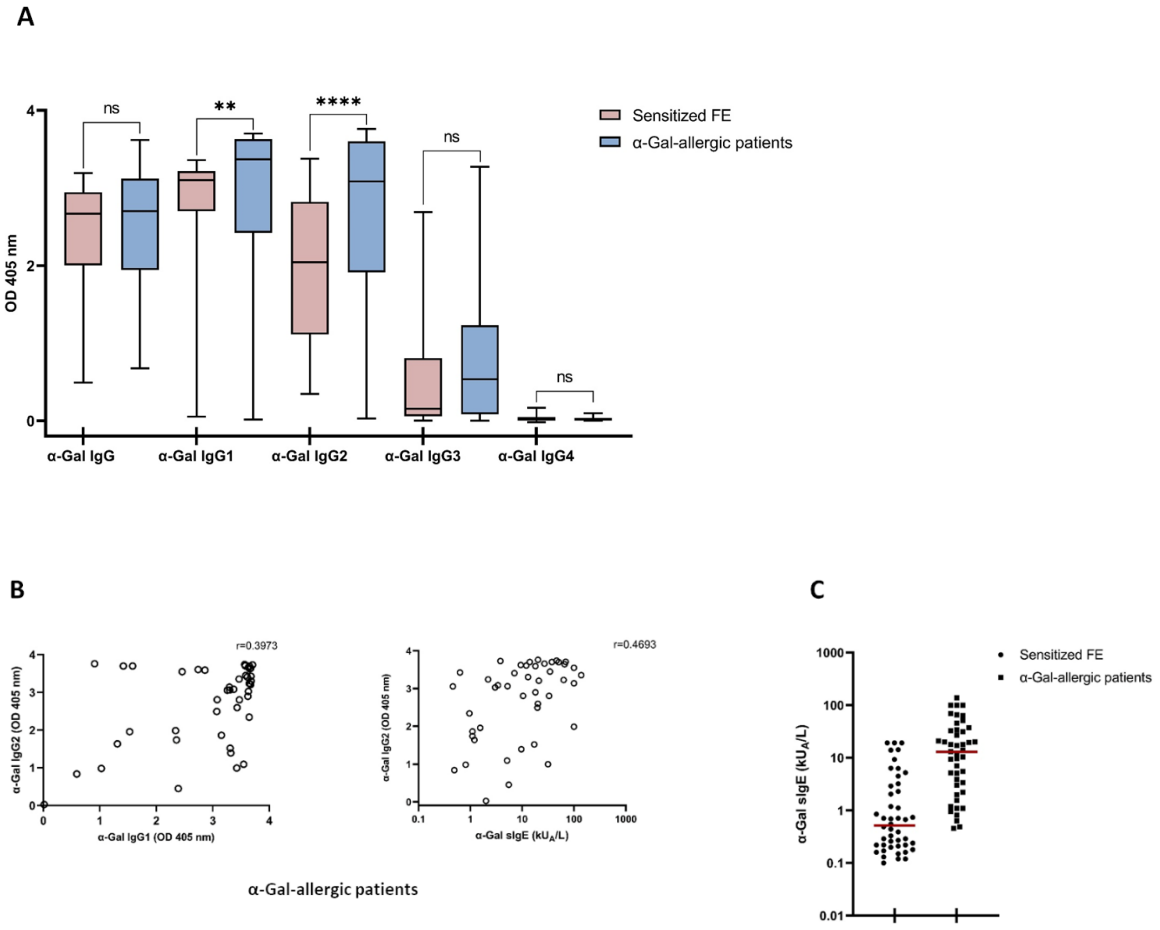


Fig 3. α-Gal-allergic patients have higher levels of IgG1 and IgG2 to α-Gal-HSA compared to sensitized forestry employees: A. Box-plot showing levels of IgG and IgG subclasses directed towards α-Gal in sensitized forestry employees (FE) (N=46) and α-Gal-allergic patients (N=45), Mann-Whitney test showing significance of comparison (ns: not significant, ** p<0.01, **** p<0.0001). Whiskers extend to minimum and maximum values, median displayed as line within each box. **B.** Spearman correlation (r) between levels of α-Gal IgG1 and IgG2 (left; r=0.3973, p<0.01), and levels of α-Gal sIgE and α-Gal IgG2 in α-Gal-allergic patients (right; r=0.4693, p<0.001). **C.** α-Gal-specific IgE levels (kUA/L) in sensitized employees (FE) (N=46) and α-Gal-allergic patients (N=45) as determined in ImmunoCAP, bar shows median levels (left).

IV α-Gal-allergic patients have a different IgG antibody profile than fish-allergic patients

In order to compare the IgG profiles of our two cohorts, the high-risk population of forestry employees and the meat-allergic patients, to a cohort of patients with an unrelated food allergy, we analyzed the sera of a group of fish-allergic patients (Table II).

Table II.

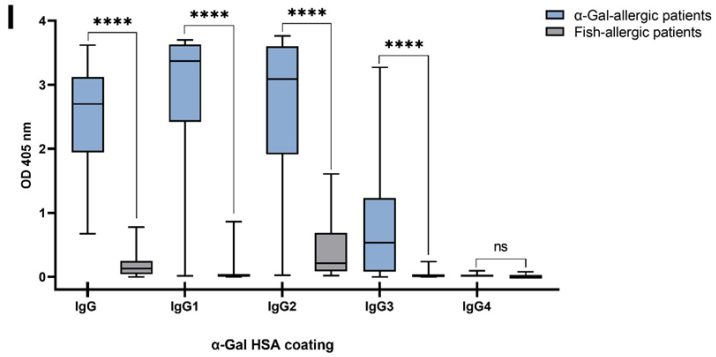
	α-Gal-allergic	Fish-allergic	Sensitized FE	Non-sensitized FE
No. (N)	45	22	46	173
Sex (male%)	29/45 (64%)	18/22 (82%)	45/46 (98%)	166/173 (96%)
Age (yrs)	54 (13 – 80)	12 (7 – 35)	43 (19 – 63)	45 (19 – 63)
sIgE (kUA/L)	12 (0.46 – >100)	10.40 (6.5 – >100)	0.52 (0.1 – 19.3)	<0.1
Total IgE (kU/L)	190 (8.7 – 4993)	828 (48 – 2297)	271.5 (26.5 – 2844.4)	78 (0 – 2115.1)

N=number, yrs=years, FE=forestry employees; age/sIgE/total IgE: median with range

In a first step, we compared meat-allergic patients with fish-allergic patients. IgG and IgG subclass profiles against α-Gal and a heated cod extract, containing native parvalbumin as main component, were compared between both patient groups (Fig. 4A). Modest levels of IgG and IgG2 were detected in fish-allergic patients, whereas α-Gal-allergic patients had significantly higher levels of antibodies of all subclasses, except IgG4 (Fig. 4A, upper panel).

A

I


α-Gal-allergic patients
Fish-allergic patients
OD 405 nm
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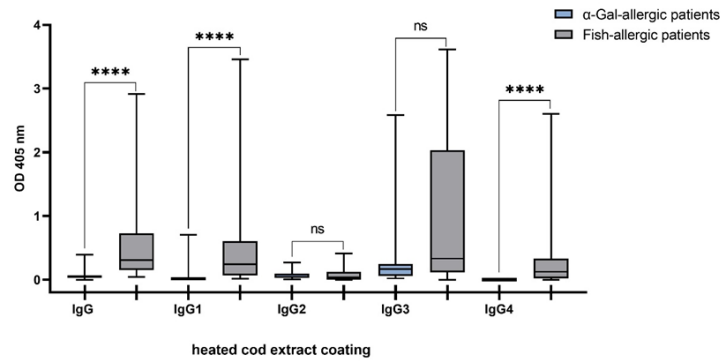
ns
IgG
IgG1
IgG2
IgG3
IgG4
α-Gal HSA coating

II


α-Gal-allergic patients
Fish-allergic patients
OD 405 nm
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IgG
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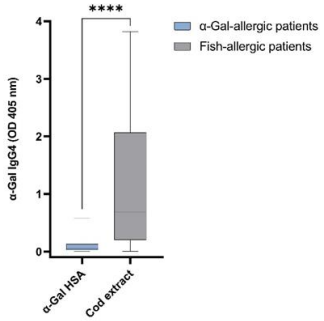
IgG3
IgG4
heated cod extract coating

B


α-Gal-allergic patients
Fish-allergic patients
α-Gal IgG4 (OD 405 nm)
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α-Gal HSA
Cod extract

C

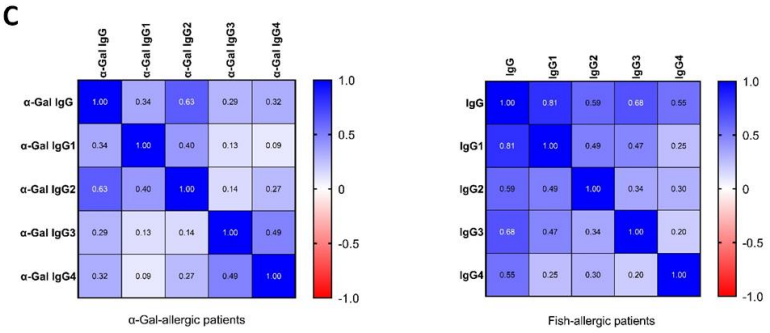
α-Gal IgG
α-Gal IgG1
α-Gal IgG2
α-Gal IgG3
α-Gal IgG4
1.00 0.34 0.63 0.29 0.32
0.34 1.00 0.40 0.13 0.09
0.63 0.40 1.00 0.14 0.27
0.29 0.13 0.14 1.00 0.49
0.32 0.09 0.27 0.49 1.00
1.0
0.5
0
-0.5
-1.0
α-Gal-allergic patients
IgG
IgG1
IgG2
IgG3
IgG4
1.00 0.81 0.59 0.68 0.55
0.81 1.00 0.49 0.47 0.25
0.59 0.49 1.00 0.34 0.30
0.68 0.47 0.34 1.00 0.20
0.55 0.25 0.30 0.20 1.00
1.0
0.5
0
-0.5
-1.0
Fish-allergic patients

Fig 4. α-Gal-allergic patients have a significantly different IgG profile than fish-allergic patients: A. Box plot comparing IgG and IgG subclass levels directed against α-Gal (upper panel) and heated cod extract (lower panel). Upper panel: α-Gal-allergic (N=45) and fish-allergic patients (N=20); lower panel: α-Gal-allergic (N=25), fish-allergic patients (N=22). Mann-Whitney comparison of antibody levels between groups (**** $p<0.0001$, ns: not significant). Whiskers extend to minimum and maximum values, median displayed as line within each box. **B.** Due to low abundance of IgG4, a more sensitive assay was used to specifically compare IgG4 levels among patients using lower serum dilution, overnight incubation and an amplifying detection system. Mann-Whitney test showing significance of difference (**** $p<0.0001$). **C.** Spearman correlation (r) matrix showing relatedness of antibody levels among IgG and IgG subclasses to α-Gal in α-Gal-allergic patients, N=45 (left); IgG and IgG subclasses to heated cod extract in fish-allergic patients, N=22 (right).

In a complementary experiment, we determined IgG and IgG subclass levels against cod in both patient groups (Fig. 4A, lower panel). As expected, fish-allergic patients showed higher IgG responses to cod than α-Gal patients, but this was highly significant only for IgG, IgG1 and IgG4. Using a high sensitivity assay for the detection of IgG4, we could confirm the very low IgG4 response to the carbohydrate α-Gal in meat-allergic patients compared to high IgG4 levels to protein allergens in fish-allergic patients, as already described by others (30, 32) (Fig. 4B). Furthermore, IgG, IgG1 and IgG2 levels to α-Gal are much higher in meat-allergic patients than respective antibodies to cod in fish-allergic patients (Fig. S3).

A Spearman correlation matrix of IgG subclasses among α-Gal-allergic patients shows a moderate correlation between IgG and IgG2 levels (r=0.63), and a weak correlation between IgG1 and IgG2 levels (r=0.40) (Fig. 4C). In fish-allergic patients, the strongest correlation is seen between IgG and IgG1, followed by IgG3, IgG2 and IgG4 (Fig. 4B). Overall, IgG and IgG subclass levels to α-Gal seem to be less correlated than corresponding antibodies directed at cod protein in the fish-allergic patient group.

V. Forest service employees develop a strong IgG response to α-Gal, compared to fish-allergic patients

IgG subclasses with highest antibody levels, IgG1 and IgG2, were compared among the four groups: α-Gal-allergic patients, sensitized forestry employees, non-sensitized forestry employees and fish-allergic patients (Fig. 5). α-Gal-allergic patients had the highest antibody levels, followed by sensitized and non-sensitized forestry employees, whereas fish-allergic patients showed very low levels of IgG, dominated by antibodies of the IgG2 subclass (Fig. 4A and 5). Most importantly, levels of anti-α-Gal IgG1 and IgG2 responses of non-sensitized forestry employees are significantly higher than that of fish-allergic patients, pointing to a continuous stimulation of IgG immune responses to α-Gal by repeated tick bites. In line with this observation, forestry employees reporting tick bites during the year of sample collection had significantly higher levels of IgG1 and IgG2 antibodies to α-Gal than employees without a history of tick bite within that year (Fig. S4).

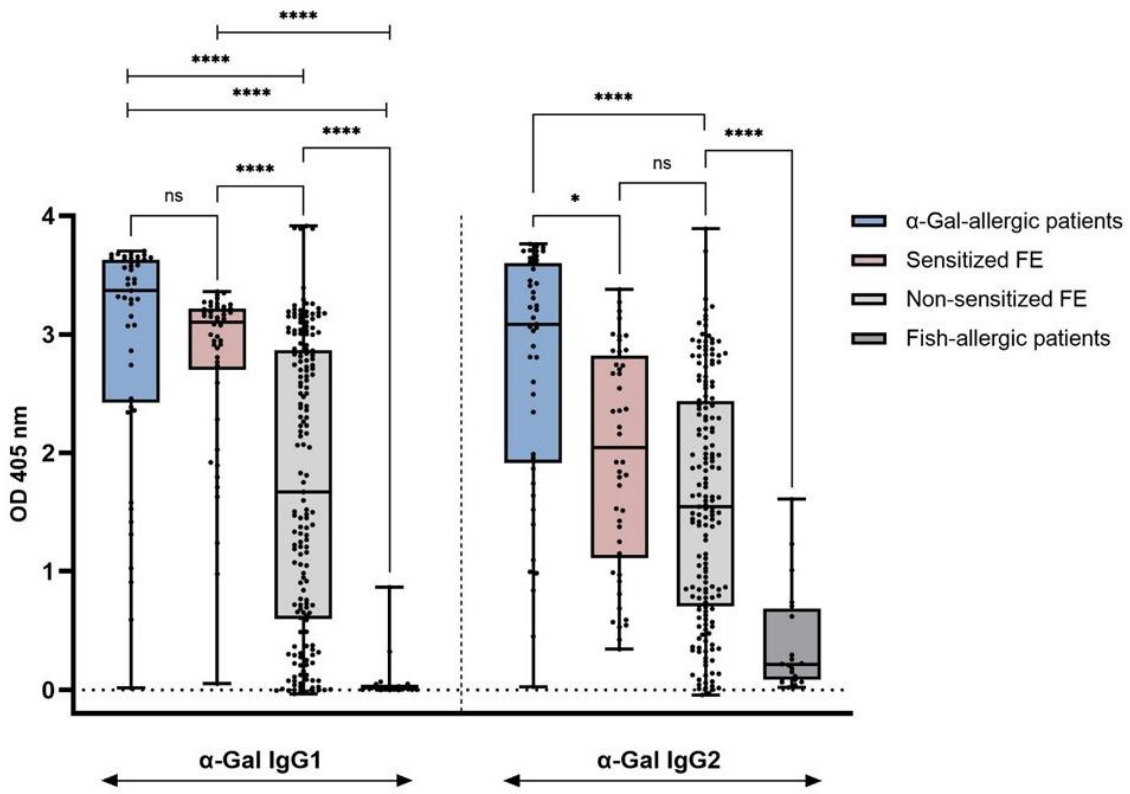

Fig 5. IgG1 and IgG2 levels to α-Gal are significantly different across groups: Box plot showing α-Gal IgG1 and IgG2 levels in sensitized forestry employees (FE) (sIgE ≥0.1 kUA/L, N=46), non-sensitized forestry employees (FE) (sIgE <0.1 kUA/L, N=173), α-Gal-allergic (0.49 – >100 kUA/L, N=45) and fish-allergic (6.5 – >100 kUA/L; N=20) patients. Kruskal-Wallis with multiple comparison test (* p<0.01, **** p<0.0001, ns: not significant). Whiskers extend to minimum and maximum values, median displayed as line within each box.

DISCUSSION

The α-Gal syndrome is considered an allergic disease that is mediated by tick bites. Ticks have been repeatedly shown to contain the α-Gal epitope (16, 17, 38), although the source of the carbohydrate, either endogenously produced or originating from residual host blood, is still a matter of debate. sIgE directed at α-Gal are associated with tick bites (39) and higher sensitization prevalence rates are reported in tick-endemic regions and in rural populations, as well as in individuals with an occupational outdoor activity, such as forestry employees (8, 24-26, 28, 40, 41).

In the present study, we retrospectively investigated sIgE levels in a high risk cohort consisting of 219 forest service employees. Using a cutoff of >0.1 kU_A/L, we found a sensitization rate of 21%. sIgE levels were low, which is in line with previous findings in high risk populations. The sensitization rate in forestry employees in Luxembourg is lower than in southwest Germany and Kentucky, 35 and 39% respectively (24, 25), but higher than in forestry employees in Spain (15%) (28). The prevalence of anti-α-Gal sIgE in the European general population varies from 4% to 8.1 %, based on a cutoff of 0.1 kU_A/L (27-29), confirming the higher prevalence rate in populations with dominant outdoor activity.

The main objective of our study was the analysis of IgG and IgG subclass profiles in a high risk cohort and to compare them with IgG profiles of meat-allergic patients and patients with a non α-Gal-related food allergy. Although previous studies have analysed IgG and IgG subclass profiles in meat allergic patients and healthy controls (30-32, 35), our study is the first to investigate IgG responses to α-Gal in a high risk population of forestry employees. Employees with sIgE to α-Gal were found to have significantly higher levels of IgG, IgG1, IgG2 and IgG3 than non-sensitized participants. Furthermore, forestry employees, sensitized and non-sensitized, had significantly higher levels of anti-α-Gal IgG1 and IgG2 antibodies than fish-allergic patients, suggesting a continuous stimulation of IgG responses by repetitive tick bites.

Notably, fish-allergic patients displayed IgG2 antibodies against α-Gal, but no relevant IgG1 or IgG3 responses. Dominant IgG2 responses to carbohydrates in general (42, 43) and specifically to α-Gal have been found earlier (34, 44) and they have been confirmed in blood donors and non-allergic controls (30-33, 35). Anti-α-Gal IgG2 are hypothesized to originate from stimulation by gut microbiota Galili (18), although the presence of α-Gal on gut bacteria has recently been questioned (45). In contrast, in meat-allergic patients and in sensitized forestry employees, anti-IgG responses to α-Gal are dominated by IgG1 whereas in non-sensitized employees, there is no significant difference between levels of IgG1 and IgG2. Taken together, IgG responses in the different cohorts suggest that tick bites favor IgG1 responses and stimulate existing IgG2 antibody responses. Tick saliva contains immunomodulatory molecules known to shift the immune response to a Th2 type (46). Consequently, high levels of inflammatory IgG1 antibodies are to be expected upon repeated tick bites and a Th2 environment would promote a direct switch from IgG1 to IgE antibodies.

In a recent study by Joral et al, IgG against α-Gal were quantified in meat-allergic patients, in individuals bitten by ticks and in healthy controls. IgG were found to be elevated in meat-allergic patients and in subjects bitten by ticks, and the quantification of IgG against α-Gal was proposed as a prognostic marker for developing mammalian meat allergy (37). In our study, many employees were employed for more than 10 years without developing a sensitization to α-Gal, hypothesizing that the different IgG subclasses may be relevant for the induction of sensitization or tolerance and that a high IgG2/IgG1 ratio may be protective.

Another important finding of our study is that fish-allergic patients showed a strong IgG4 response to fish extract, whereas IgG4 levels in sensitized forestry employees and meat-allergic patients were barely above detection level. This finding is in accordance with previous studies comparing IgG4 responses to α-Gal in meat-allergic patients with IgG4 directed at Mal d 1 in patients with birch pollen-

related apple allergy (30) and at salmon parvalbumin in fish-allergic patients (32). Thus, allergic as well as non-allergic responses to α-Gal are characterized by absence of specific IgG4 antibodies. Another important observation relates to the fact that in meat-allergic patients and in forestry employees, IgG antibody levels directed at α-Gal are significantly higher than IgG directed at fish extract in fish-allergic patients (except for IgG4). In patients with sIgE to CCD, IgG responses were also dominated by IgG1, although levels were lower than IgG1 against α-Gal in meat-allergic patients (32). Whether these high antibody levels are a general characteristic for α-Gal related IgG responses remains to be confirmed.

A major strength of our study is the analysis of anti-α-Gal IgG and IgG subclass profiles in a large cohort of participants that have a high occupational risk of recurrent tick bites. The comparison of these profiles with food-allergic patients, a group allergic to meat and a group allergic to fish, allowed us to discover distinct IgG profiles in individuals bitten repeatedly by ticks. A weakness of our study is certainly the lack of clinical data of the high-risk population, as well as data on mammalian meat consumption. In a German study on forestry employees, Fischer et al. detected a total of 5 individuals with α-Gal syndrome among the study group, representing 8.6% of the group with sIgE ≥0.35 kU/L (25). When extrapolating to our cohort, about 2-3 individuals may potentially be affected by meat-allergy.

In conclusion, our study shows elevated levels of IgG directed against α-Gal in a high risk cohort of forestry employees with recurrent tick bites, providing evidence for a continuous stimulation of α-Gal-related immune responses by ticks. Whereas IgE-sensitized employees present an IgG response dominated by IgG1, non-sensitized individuals have a more equilibrated IgG1 and IgG2 response. A hallmark of sensitization to α-Gal is the lack of a marked IgG4 response to α-Gal, distinguishing red meat allergy from protein-related food allergies.

SUPPLEMENTARY DATA

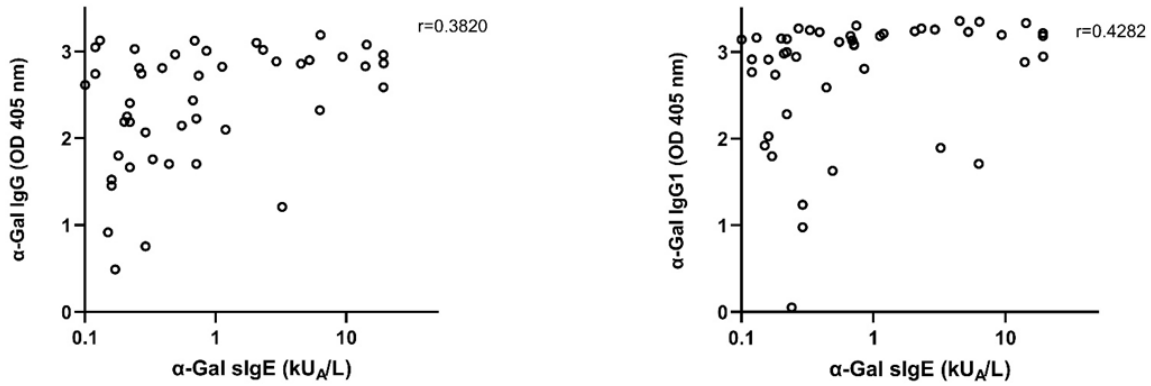

Fig S1. α-Gal IgG weakly correlated with sIgE levels in the sensitized forestry employees: Spearman correlation(r) of α-Gal sIgE levels to α-Gal IgG (left; r=0.3820, *p*<0.01) and to α-Gal IgG1 (right; r=0.4282, *p*<0.01) in sensitized forestry employees.

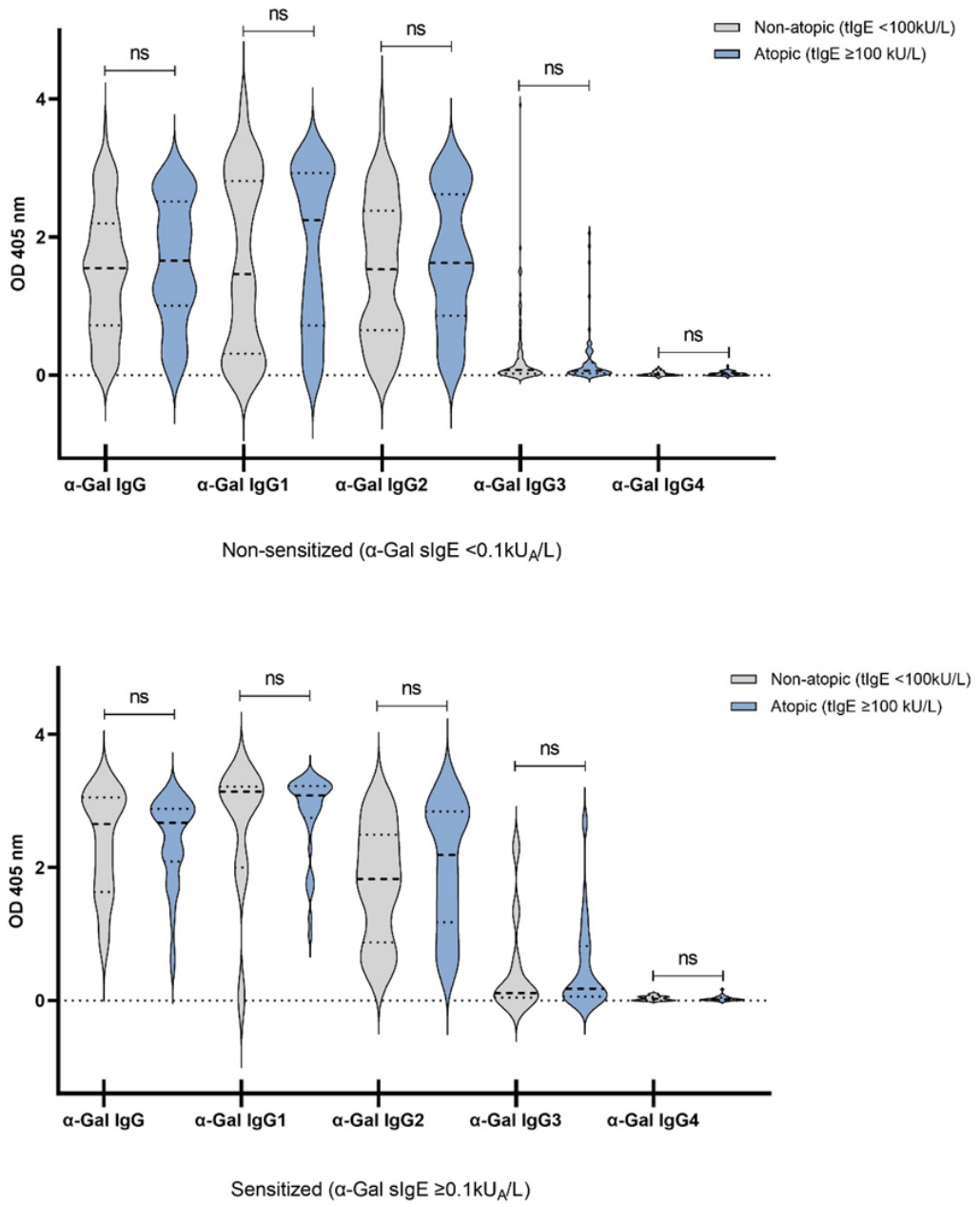

Fig S2. No significant correlation between atopy (total IgE ≥100 kU/L) and increased levels of α-Gal IgG among forest employees: Violin-plot showing α-Gal IgG/subclass levels in forestry employees (non-sensitized *versus* sensitized). No significant difference (Mann-Whitney comparison, ns: not significant) observed in α-Gal IgG levels among non-sensitized (top; N=173, α-Gal sIgE <0.1 kU$_A$/L) and sensitized (bottom; N=46, α-Gal sIgE ≥0.1 kU$_A$/L) cohort when segregated based upon the atopic status (total IgE ≥100 kU/L).

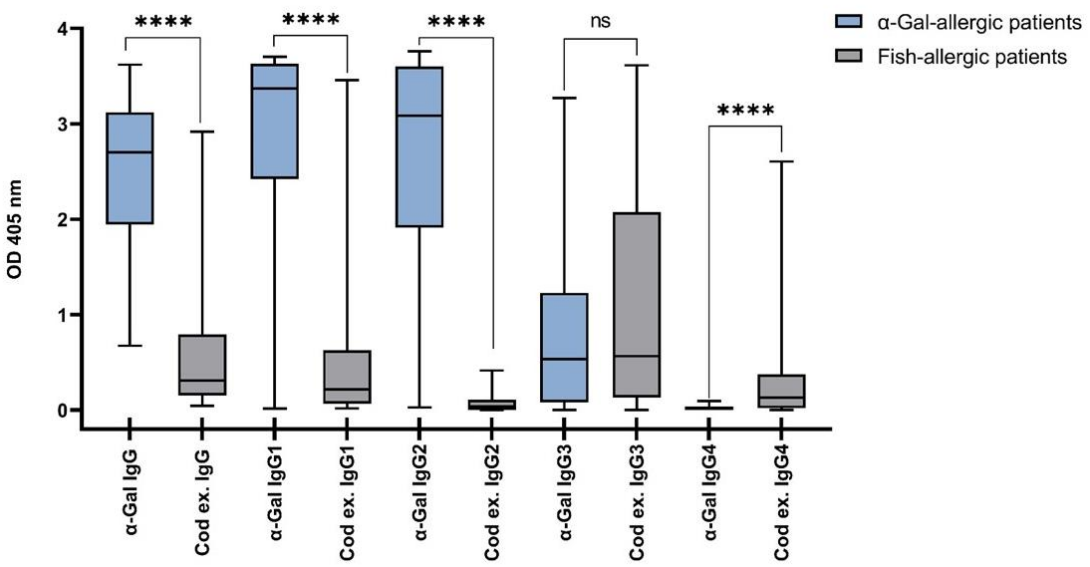

Fig S3. α-Gal-allergic patients have significantly higher IgG levels to α-Gal compared to fish-allergic patients to cod extract: Box plot showing IgG/subclass levels in α-Gal-allergic patients to α-Gal HSA (N=45) and IgG/subclass levels to cod extract in fish-allergic patients (N=22). Mann-Whitney comparison shows significant difference in IgG/subclass levels between groups to the respective allergen/extract (**** p<0.0001, ns: not significant).

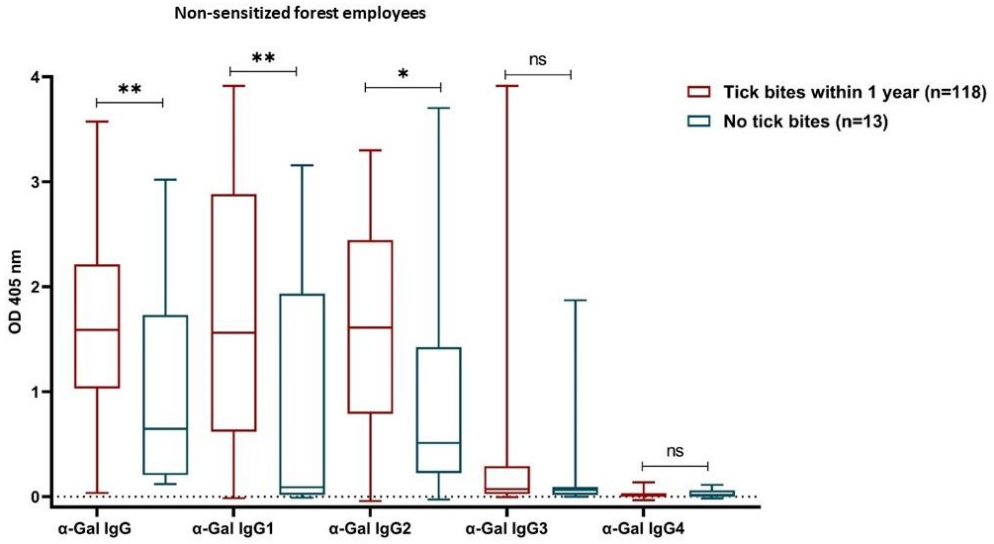

Fig S4. α-Gal IgG/subclass levels in non-sensitized forestry employees reporting no tick bites (n=13) *versus* those bitten within one year before sample collection (n=118). Mann-Whitney comparison shows significant difference in α-Gal IgG, IgG1, and IgG2 levels (** p<0.001, * p<0.01, ns: not significant).

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CHAPTER II

CHAPTER II: Mouse blood protein survives degradation in *Ixodes ricinus* ticks after moulting and upon starvation up to five months

N. Chakrapani,..., C. Hilger (2022). Mouse blood protein survives degradation in *Ixodes ricinus* ticks after moulting and upon starvation up to five months. *Manuscript in preparation*

Author contributions:

In this study, I have prepared the protein extracts, analyzed and interpreted the data and wrote the manuscript with inputs from CH. MW provided longitudinal samples of larvae and nymphs and AL has processed the samples and acquired MS/MS data. GD conducted proteomics analysis and FJ participated in data interpretation. CH, MO and UM conceptualized and supervised the study.

MANUSCRIPT

Mouse blood protein survives degradation in *Ixodes ricinus* ticks after moulting and upon starvation up to five months

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ABSTRACT

Background: α-Gal allergy is characterized by a delayed allergic response occurring upon consumption of mammalian meat and by-products. Numerous studies have confirmed hard ticks to be the source of sensitization to α-Gal. The carbohydrate has been detected in tick midgut, salivary gland, and ovaries by immunohistochemistry. However, the source of these sensitizing molecules in ticks remains open to speculation as α-Gal could originate from either endogenous production or residual host blood protein.

Objective: To study kinetics of host blood protein degradation in fully engorged *Ixodes ricinus* larvae upon prolonged starvation after moulting into nymphs.

Method: 1200 *Ixodes ricinus* larvae were fed on BALB/c mice under laboratory conditions. Fully engorged larvae were allowed to moult into nymphs and subsequently starved for 0, 10, and 20 weeks. Protein extracts from starved nymphs were prepared in PBS and analysed via LC-MS/MS mass spectrometry.

Results: Twenty-one percent of the larvae survived and moulted into nymphs. Approximately 17 to 70 nymphs were analysed per time point and 50 – 100µg of protein was obtained. More than 500 unique tick proteins were detected in unfed larvae and 0, 10, 20 week-old nymphs. Overall, similar global tick protein content was observed across all time-points post-moulting, but a distinct protein profile emerged immediately after moulting (0 week). In total, peptides originating from 21 mouse proteins were detected, out of which 9 proteins were detected across all time-points post-moulting. Most proteins were secreted or extracellular; however, proteins originating from cellular compartments such as cytoplasm, nucleus, cytoskeleton etc. were detected as well. Serum albumin, haemoglobin, serine protease inhibitor and serotransferrin were detected in high quantities at week 0 and were still detectable at week 20. More than seventy percent sequence coverage was observed for mouse serum albumin, pointing to the presence of intact protein at all stages.

Conclusion: Under controlled conditions, host blood proteins survive moulting and are passed onto the next developmental stage in *Ixodes ricinus* ticks, from larvae to nymphs. These proteins are detectable up to five months post-moulting and could be a sensitization source, triggering allergy to α-Gal.

INTRODUCTION

Ticks are important vectors of various diseases and have long been subject of much pharmacological interest. Red meat allergy is a recent addition to the list, where bites by hard ticks lead to sensitization and production of IgE antibodies towards the carbohydrate galactose-α-1,3-galactose (α-Gal) (1-10). Symptoms are characterized by a delayed form of allergic reaction occurring 2-6 hours after consumption of mammalian meat or dairy products such as milk and cheese (11-13), additionally patients can experience an immediate reaction upon intravenous administration of biologicals containing α-Gal (14). Humans, apes, and old-world monkeys lost α-1,3-galactosyltransferase (α-1,3-GT) activity due to inactivation of the GGTA 1 gene owing to a frameshift mutation (15, 16). They thus lack the carbohydrate, enabling them to develop IgG antibodies, which is believed to have provided them an evolutionary advantage by conferring protection against α-Gal expressing pathogens. In 2009, Van Nunen et al. first reported a link between tick bite exposure and red meat allergy followed by similar reports from southeastern US where bites by *Amblyomma americanum* (lone star tick) a locally abundant deer tick were associated with sensitization to α-Gal (17, 18). Since then bites from various tick species all over the world have been correlated with increase in specific IgE against α-Gal in humans, including *Ixodes ricinus* (Europe), *Amblyomma sculptum* (Brazil), *Ixodes scapularis* (USA), *Amblyomma cajennense* (Costa Rica, Panama and Ivory Coast), *Ixodes holocyclus* (Australia), *Haemaphysalis longicornis* (Korea, Japan) etc. (1, 4, 8, 10, 19-23). As red meat allergy is triggered by tick bites, it is now considered as the first vector borne allergic disease to be reported.

α-Gal carrying proteins have been detected in the saliva/salivary gland extract of *Amblyomma americanum*, *Ixodes scapularis*, *Amblyomma sculptum*, *Haemaphysalis longicornis* and *Hyalomma marginatum* (24, 25)(Chinki 2016, Sharma 2021 etc.). *I. ricinus* is the major causative vector of α-Gal allergy in Europe with cases reported in Sweden, Germany, Luxembourg, Italy, France, Switzerland, Spain and Norway (1, 8). Hamsten et al. (2013) first detected α-Gal in the gastrointestinal tract of *I. ricinus* using patient sera and a polyclonal mouse anti-α-Gal antibody (26). A recent histology study by Fischer et al. (2020) showed the presence of α-Gal in the salivary gland, hemolymph and midgut of fed and unfed female adult *I. ricinus* (10). N-glycan profiling of salivary glands of male and female *A. americanum* by Park et al. (2020) found an increase in α-Gal epitopes over time after onset of feeding (27). Crispell et al. (2019) detected glycoforms of α-Gal in N-glycan profiling of saliva from partially fed *A. americanum* and *I. scapularis,* and in unfed salivary gland from *I. scapularis* (4). Prevailing theories on occurrence of α-Gal include – metabolic incorporation of host glycans into ticks, presence of residual glycan-carrying-molecules from a previous blood meal, α-Gal contribution by tick microbiome or endogenous production by ticks (28). A recent study on transcriptomic and proteomic annotation of naïve *I. ricinus* midgut by Cramaro et al. found it to be devoid of α-1,3-GT activity, in addition tick database searches were negative for α-1,3-GT activity (29). The lack of α-1,3-GT is also confirmed by Cabezas-Cruz et al. (2018), who then investigated if *Ixodes scapularis* β-1,4-galactosyltransferase (β-1,4-GT) and α-1,4-galactosyltransferase (α-1,4-GT) would play a role in α-Gal synthesis. They were able to confirm production of α-Gal by immunofluorescence using two heterologous models, *E. coli BL21* and human HL-60 cells, expressing β-1,4-GT and α-1,4-GT (30). Another study by Sharma et al. (2021) analyzed the role of α-D-galactosidase and β-1,4-GT in a tick model of RNA silencing. The authors report a decrease in galactose metabolism and transport in *A. americanum* as well as a significant reduction in α-Gal levels in silenced tissue, whereas no significant difference was observed in α-Gal levels upon β-1,4-GT inactivation, suggesting that β-1,4-GT does not contribute to endogenous α-Gal production (25). The origin of α-Gal in ticks is still a matter of debate. So far, there is yet a lack of experimental evidence on metabolic recycling of host α-Gal and on a putative role of the tick microbiome in α-Gal sensitization.

Ixodes ticks are obligate ecto-parasites on vertebrates, feeding once at each developmental stage of their life cycle. They have a robust survival mechanism, with a lifespan ranging from 6 months to 2-3 years. They can consume up to 100 times their body weight of blood and go on prolonged starvation periods extending many months (31); therefore, it is tempting to speculate that α-Gal originating from residual mammalian host blood could constitute an important sensitization source. Indeed, Wickramasekara and colleagues (2008) identified residual mammalian host blood components in *I. scapularis* and *A. americanum* when fed on rabbit, sheep and mouse, and Laskay et al. (2013) analyzed the presence of host blood in *I. scapularis* over a time course after molting, pointing towards long retention of host blood in these tick species (32, 33). Using a proteome profiling technique, Önder et al. detected mammalian fingerprints in *I. scapularis* up to 6 months after feeding (34). As mammalian protein could be passed into humans via tick saliva or by regurgitation during feeding, residual host protein is a plausible source of sensitization to α-Gal.

The objective of the present study was to analyze for the first time the content of mouse protein in *I. ricinus*, a tick playing a major role in sensitization to α-Gal in Europe. The longitudinal approach of our study allowed us to detect and identify mouse proteins at different time points after feeding, molting and up to several months of starvation, and to relatively quantify the respective amounts of mouse proteins.

MATERIALS AND METHODS

Ethics statement

All animal experiments were performed in compliance with the German animal welfare law. The animals were housed and handled in accordance with the good animal practice as defined by FELASA. All animal experiments were approved by the responsible state office (Baden-Württemberg, Regierungspräsidium Stuttgart) under permit number A 388/14 PA.

Tick feeding and starvation

Ixodes ricinus larvae were purchased from Insect Services (Berlin, Germany) and maintained at the Department of Parasitology, University of Hohenheim, where feeding and subsequent starvation was performed. Twelve hundred larvae were fed on 20 BALB/c mice (20 larvae/mouse) in three independent runs at an interval of 10 days. Briefly, 20 tick larvae were placed on a BALB/c mouse that was in an empty cage. Larvae that fell off the mouse were placed back with a fine paintbrush until no larvae could be observed off the host. The mice were subsequently transferred to a modified cage. For the duration of tick feeding the BALB/c mice were kept individually above water on elevated perforated steel plates. Engorged larvae, which detached after 3-6 days of feeding, were recovered from water during the daily inspection. Larvae were then stored in glass vials with perforated snap-on lids which were placed in plastic boxes at room temperature with high relative humidity (>90 %, established by saturated $MgSO_4$ solution in the boxes). They were exposed to a natural light:dark cycle until moulting. After moulting, a part of the nymphs was transferred to plastic vials, the opening covered with a nylon cloth and sealed with a perforated cap and shipped for analysis. The remaining nymphs were stored under the same conditions as described above and allowed to starve for a period of 10 and 20 weeks respectively before shipment. Nymphs were stored at −80°C upon arrival until processing.

Protein extraction and analysis

Due to the individual development time of the nymphs (approximately eight weeks between first and last moulting nymph), a different number of nymphs were available at the respective time points. Protein extracts were prepared by pooling 17 – 32 ticks per time-point in order to reduce biological variation. Two and three biological replicates were prepared for samples at 10 weeks and 20 weeks respectively whereas two technical replicates were analysed for 0 week and the unfed larvae samples. The analysis time points were labeled 0 months (0M), 2.5 months (2.5M) and 5 months (5M). A pool of unfed larvae (>50) served as negative control.

Ticks were transferred to 2ml low-protein binding tubes (Eppendorf) and crushed in PBS (pH 7.4, Lonza) supplemented with protease inhibitor (Roche) using 5mm steel beads at 2x2 minute cycles of 30 Hz/sec on a TissueLyser (Qiagen). Alternatively, ticks were transferred to a single use lysing matrix H tubes (MP Biomedicals) and crushed in PBS supplemented with protease inhibitor at 30 Hz/sec in 5-10 minute cycles until completely solubilized (3-4 cycles). Crushed extract was incubated at 4°C for 1 hour with constant shaking, centrifuged at 15000xg for 10 minutes and supernatant collected. Supernatant was re-centrifuged to pellet any remaining debris and stored in low-protein binding tubes. In case of low recovery pellet was re-extracted in PBS by a second incubation at 4°C for 1 hour and the supernatant was combined. Protein concentration was determined at A205 nm on NanoDrop (ThermoFischer Scientific) and sample quality analyzed on mini-PROTEAN TGX pre-cast gel (Biorad) by silver-stain

(Pierce, ThermoFischer Scientific) as per manufacturers' instructions. Extracts were stored at -20°C until processing.

Proteomics analysis – sample processing and MS/MS data acquisition

10µg of each tick protein extract were vacuum dried and re-suspended in 20µL of 50mM ammonium bicarbonate buffer, pH 8.0. Proteins were reduced with 5 µL of dithiothreitol (200 mM) for 30 minutes at room temperature and further alkylated with 10µL of iodoacetamide (400 mM) at room temperature in the dark for 30 minutes. The remaining iodoacetamide was eliminated by the addition of 10 µL of dithiothreitol (200 mM). Proteins were captured and cleaned by using magnetic SP3 beads (Thermo Fischer Scientific) according to the Hughes et al. protocol (35). Briefly, proteins were captured on beads with acetonitrile (final concentration 1/1, v/v), then the beads were washed 3 times with a 70% ethanol solution. The beads were suspended in the proteolysis buffer (50mM ammonium bicarbonate buffer, pH 8.0) with 250ng trypsin/lysine-C mixture (1/40 ratio w/w)) and incubated overnight at 37°C. The resulting peptides were re-captured on the beads, cleaned by acetonitrile washes, and finally eluted with 2% dimethyl sulfoxide (DMSO). Eluted peptides were then vacuum dried and suspended in the MS loading buffer (H2O with 1% acetonitrile and 0.05% TFA). Sample concentrations were estimated by absorbance at 205 nm in a nanodrop (Thermo).

The peptide samples were analyzed by liquid chromatography coupled mass spectrometry (LC-MS/MS) on a Dionex U3000 RSLC system configured in column switching mode (mobile phase A: 0.1% formic acid in water and B: 0.1% formic acid in acetonitrile; loading phase: 0.05% TFA and 1% acetonitrile in water). 200ng peptides were injected and trapped onto a C_{18} trap column (PepMap 100 C18, 75µmx2cm, 3µm), and eluted onto a C_{18} analytical column (PepMap 100 C18, 75µmx15cm, 2µm) by a gradient ranging from 2% B to 35% B over 66 minutes. The MS acquisition was performed with a Q-Exactive Plus mass spectrometer (Thermo Scientific) in data-dependent acquisition mode. The MS cycle of the high-resolution survey scan was set to 70,000 (at 200 m/z) followed by the fragmentation of the top 12 most intense precursor ions at a resolution of 17,500 (at 200 m/z). Singly charged precursors were excluded and a dynamic exclusion of the already fragmented precursor ions was set to 30s.

Data processing and bioinformatics analyses

The recorded spectra were automatically analysed using the MaxQuant software package (Version 2.0.1.0). The data was analyzed using the combination of the *Mus musculus* (UP000000589, 55315 entries), *Ixodes scapularis* (UP000001555, 20486 entries), and a database containing common contaminants (MaxQuant standard library). For the data interpretation, the variable modifications were set to methionine oxidation and N-terminal acetylation, while the fixed modifications were set to carbamidomethylation of cysteins. The minimal peptide length was set to 8 and the maximum to 25 amino acids length. The number of missed cleavages was set to 5 with the enzyme set to trypsin/P. Related samples were analysed with match between runs with a matching window of 0.7 minutes. Label-free and iBaq quantification were activated. The FDR was set to 1% for peptides and proteins. The processed protein and peptide was further processed and analysed for statistical significance using R (4.2.0).

RESULTS

Experimental design

The aim of this longitudinal study was to analyse the presence of mouse blood proteins in *Ixodes ricinus* nymphs under controlled conditions, encompassing feeding at larval stage, moulting into nymphs and starvation for a prolonged time. The experimental setup of the study is summarized in figure 1, unfed *Ixodes ricinus* larvae were allowed to feed on BALB/c mice until they were fully engorged and fell off the host. A total of 1200 larvae were fed on 20 mice in three different experimental runs and 249 living nymphs were recovered after moulting and starving. A first batch of nymphs was processed directly after moulting (n=17), 2 batches of nymphs were processed after 10 weeks (N=23, N=32) of starvation, and the last group was processed in 3 batches after 20 weeks of starvation (N=19, N=25, N=29). For each batch, ticks were pooled for protein extraction, and an average protein yield of 2.6 µg/nymph (range 2.4 to 3.1 µg/nymph) was obtained. In addition, a preparation of unfed larvae (N>50) served as control. Analysis was performed in three experimental runs, experiment 1 and 2 serve as a first run to get an insight into the presence of the mouse blood proteins whereas experiment 3 was a rerun of all samples together in order to minimize non-biological variation and to compare samples across different time points.

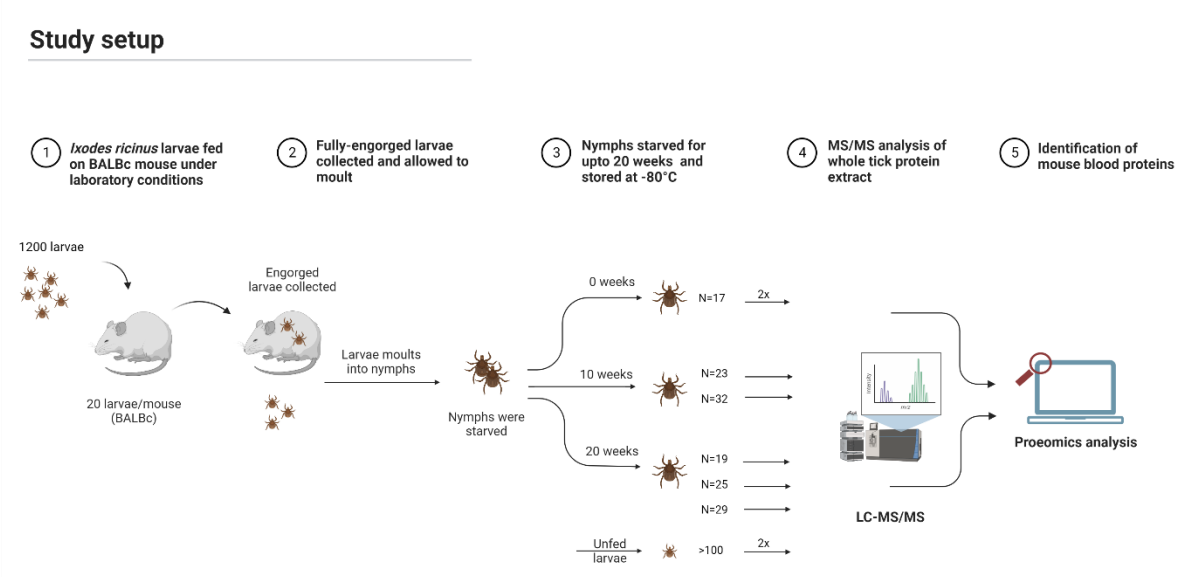
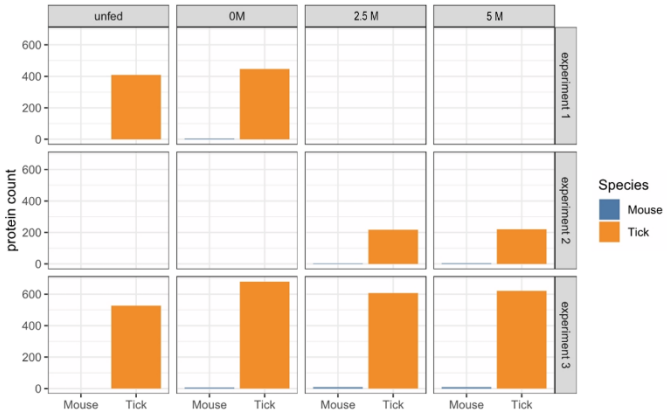


Figure 1. A schematic overview of larval feeding and proteomics analysis of the moulted nymphs.

Multiple mouse blood proteins detected after moulting and starvation

To detect mouse blood protein, tick samples were analysed using a combined mouse, tick and human proteome (UP000005640) database and a database for the common contaminants. Peptides annotated as “human”, “human mouse”, “human mouse tick”, and “common contaminations” were considered as contaminations and removed from the analysis. Moreover, all identifications which were not unique to mouse were ignored.

A



B

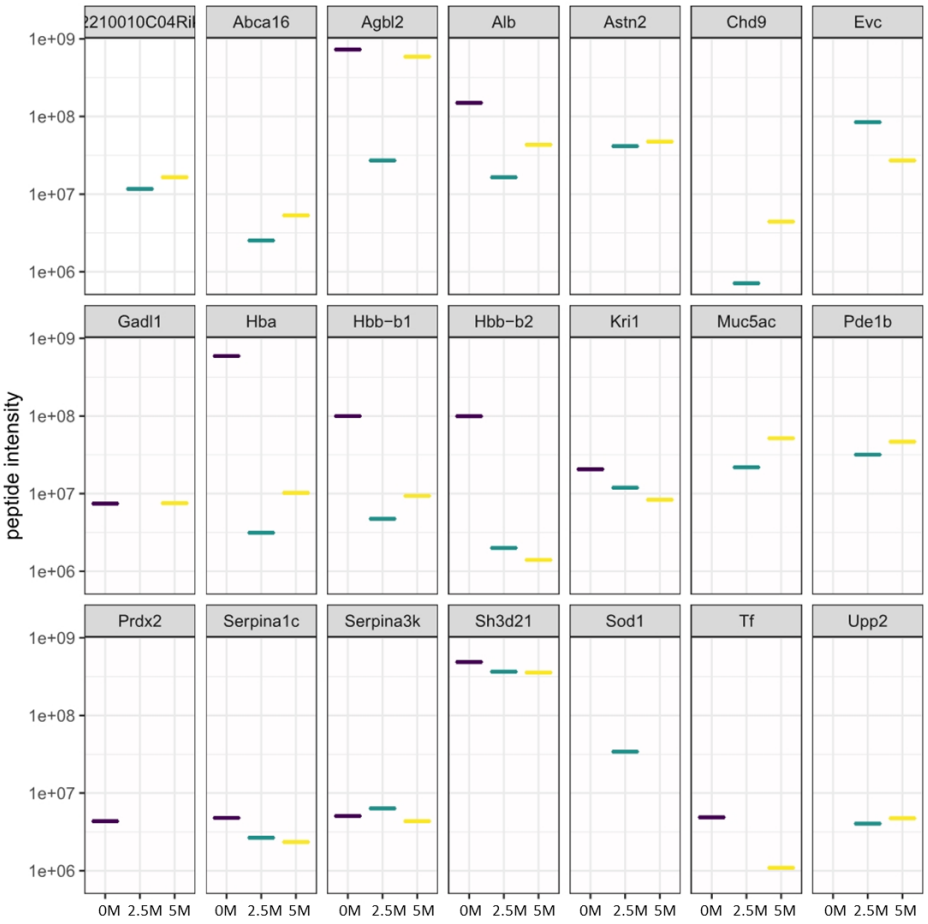


Figure 2. Multiple mouse blood proteins detected in nymphs after moulting and upon starvation up to twenty-four weeks: A. Mouse and tick proteins detection in three experimental runs. Experiment 1: unfed larvae (unfed) and nymphs directly after moulting (0M); experiment 2: nymphs after 10 weeks (2.5M) and 20 weeks (5M) of starvation; experiment 3: mouse and tick proteins detected in all samples in one run – unfed larvae (unfed), nymphs after moulting (0M), after 10 weeks (2.5M) and 20 weeks (5M) of starvation. **B.** Normalized bar chart showing all host-blood proteins detected at 0M/2.5M/5M in experiment 3. Y-axis - median peptide intensity.

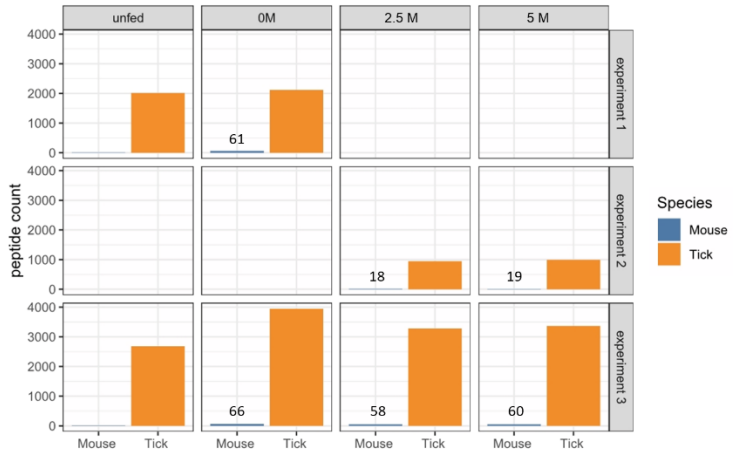
Mouse proteins are retained at nymphal stage as seen in experiments 1, 2 and 3 (figure 2A); overall, peptides from 9-10 proteins were detected in nymphs directly after moulting, 8-14 proteins after 10 weeks and 8-15 proteins after 20 weeks (figure 2A). In total, 21 mouse proteins were detected, out of which nine were present at all three time points (0M/2.5M/5M), ten could be detected at two time points, whereas two proteins could only be detected at one time point (figure 2B). Serum albumin (Alb), hemoglobin (Hba and Hbb-b1/b2), serine protease inhibitor (Serpina 3k) and serotransferrin (Tf) were present in high quantities and 40 (Alb), 34 (Hba, Hbb b1/b2), 8 (Serpina 3k), and 6 (Tf) unique peptides were detected. Most proteins were extracellular/secreted; however, proteins originating from cytoplasm, nucleus, cytoskeleton etc. were present showing a varied protein representation. A summary list of top 10 most abundant proteins is shown in Table I.

Table I. Summary list of top 10 mouse proteins detected

No.	Gene name	Protein name	Entry	Unique peptides	Subcellular location	N-linked glycosylation
1	Agbl2	Cytosolic carboxypeptidase 2	Q8CDK2	1	Cytoplasm/cytosol/cytoskeleton	-
2	Alb	Albumin	P07724	40	Extracellular/secreted	-
3	Chd9	Chromodomain-helicase-DNA-binding protein 9	Q8BYH8	2	Cytoplasm/nucleus	-
4	Hba	Hemoglobin subunit alpha	P01942	8	Extracellular/ hemoglobin complex	-
5	Hbb-b1	Hemoglobin subunit beta-1	P02088	8 (+14 shared)	Extracellular/ hemoglobin complex	-
6	Hbb-b2	Hemoglobin subunit beta-2	P02089	6 (+14 shared)	Extracellular/ hemoglobin complex	-
7	Prdx2	Peroxiredoxin-2	Q91WA1	3	Cytoplasm/Nucleus	-
8	Serpina 1c	Serine Protease inhibitor 1c	A0A0R4J0X5	3	Extracellular/secreted	3
9	Serpina 3k	Serine Protease inhibitor 3k	P07759	8	Extracellular/secreted	4
10	Tf	Serotransferrin	Q921I1	6	Extracellular/secreted	1

The analysis of the peptides by species (mouse/human/tick) shows that all samples contain a significant number of peptides of mouse origin. A total 61 and 66 mouse peptides were detected at 0 week (after moulting, 0M) in experiment 1 and 3 respectively, and 58 and 60 peptides were detected in experiment 3 samples after 10 weeks (2.5M) and 20 weeks (5M) of starvation respectively (figure 3A). We could only detect 18 and 19 peptides in the experiment 2 due to sample loss while processing.

A


unfed
0M
2.5 M
5 M
experiment 1
experiment 2
experiment 3
peptide count
4000
3000
2000
1000
0
61
18
19
66
58
60
Mouse
Tick
Species
Mouse
Tick

B


AADKDTCFSTEGPNLVTR
AETFTFHSDICTLPEK
AETFTFHSDICTLPEKEK
ALYQTEAFTADFQQPTEAK
APQVSTPTLVEAAR
AQEEVDYLEWLK
Alb
Serpina3k
Kri1
DLQILAEFHEK
DTCFSTEGPNLVTR
CCSGSLVER
CCTLPEDQR
DVFLGTFLYEYSR
ECCHGDLLECADDRAELAK
ELISELDER
ENPTTFMGHYLHEVAR
ENYGELADCCTK
FSIASNYR
GLVLIAFSQYLQK
GTFASLSELHCDK
HPDYSVSLLLR
KQTALAELVK
KYEATLEK
LCAIPNLR
LEEDVLPEMGIK
LNETQLSGR
Hbb-b1
Agbl2
LPCVEDYLSAILNR
LQTCCDKPLLK
LSQTFPNADFAEITK
LVQEVTDFAK
MFASFPTTK
SRLELLELK
TCVADESAANCDK
Hba
Sh3d21
TMEEILEGLK
TNCDLYEK
TPVSEHVTK
TVMDDFAQFLDTCCK
TYFPHFDVSHGSAQVK
VHLTDAEK
VITAFNDGLNHLDSLK
VVAGVATALAHK
YFDSFGDLSSASAIMGNAK
YFDSFGDLSSASAIMGNPK
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YNDLGEQHFK
peptide intensity
1e+09
1e+08
unfed
0M
2.5 M
5M

C

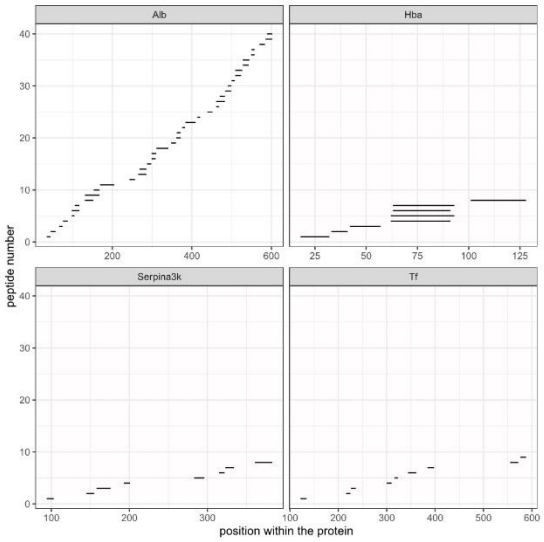

Figure 3. Large no. of mouse peptides detected in *Ixodes ricinus* nymphs – post-moulting and after starvation: A. Total number of mouse and tick peptides detected at each time point in the three experimental runs – unfed (larvae), 0M (nymphs after moulting), 2.5M (nymphs after 10 weeks of starvation), 5M (nymphs after 20 weeks of starvation). **B.** Median peptide intensity of top 49 unique mouse peptides as detected in experiment 3; gene name on top left corner. **C.** Peptide mapping of the four abundant mouse proteins detected – Albumin (Alb), Hemoglobin subunit alpha (Hba), Serine protease inhibitor A3K (Serpina3k), and Serotransferrin (Tf).

Figure 3B shows the top 49 unique mouse peptides, majority of the detected peptides came from albumin (Alb) and hemoglobin (Hba, Hbb-b1, Hbb-b2) followed by serine protease inhibitor 3k (Serpina 3k), in addition three peptides belonging to the proteins – Protein KRI1 homolog (Kri1), Cytosolic carboxypeptidase 2 (Agbl2), and SH3 domain-containing protein 21 (Sh3d21) were identified. The number of detected peptides for the three proteins were comparable over time. The intensities at 0 week (0M) include some peptides that have a 10 fold higher intensity. The per peptide intensity stayed at the same level for most peptides at 10 weeks (2.5 M) and 20 (5M) weeks.

Peptide mapping of the four most abundant proteins show high sequence coverage with N- and the C-terminal coverage for all four proteins thereby showing that the detected peptides are not a part of the stable fragment of another protein. Albumin (Alb) and hemoglobin subunit alpha chain (Hba) are extensively covered and multiple peptides broadly covering the sequences of serine protease inhibitor 3k (Serpina 3k) and serotransferrin (Tf) are shown (figure 3C).

High quantities of host blood protein retained for a long duration

Having confirmed the presence of mouse proteins in ticks at several time points after molting from larvae to nymphs, next we aimed at analysing the relative quantification of mouse and tick proteins. To compare the amounts of proteins in the different samples we used the iBAQ (intensity based absolute quantification) technique. iBAQ uses the summed intensity of the detected peptides and normalizes them to the number of detectable peptides. This allows the direct amount comparison between different proteins.

No mouse protein was detected in the unfed larvae while the average intensity of the mouse proteins in experiments 1-3 is higher than the average tick proteins, which indicates that the concentration of host blood protein is rather high compared to the total tick protein content (figure 4). Thousands of

tick peptides were identified compared to around sixty mouse peptides at each nymphal stage (experiment 1 and 3), thus indicating retention of high quantities of host blood even after prolonged starvation period.

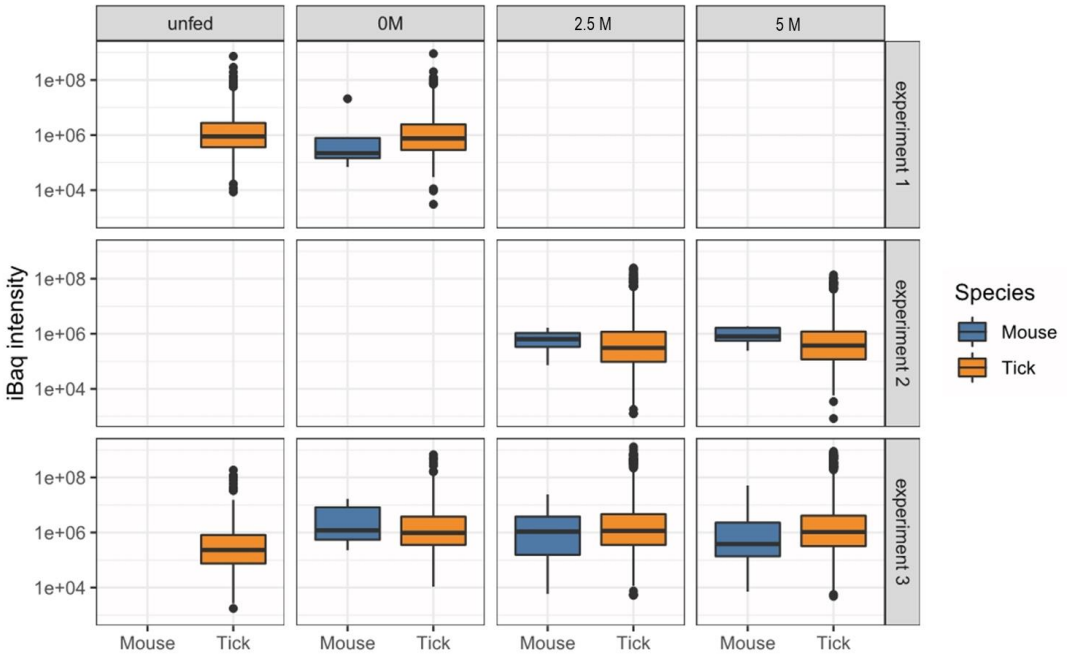

Figure 4. High quantities of mouse proteins present compared to the tick proteins: Intensity based absolute quantification (iBaq) shows high quantities of mouse blood proteins in nymphs; iBaq intensity of mouse and tick protein content in each sample after moulting (0M) and upon starvation period of 10 weeks (2.5M) and 20 weeks (5M) across three experimental runs. Y-axis: intensity-based absolute quantification (iBAQ).

Differential tick protein expression across developmental stages

The transition to a different developmental stage alters the metabolic state of the ticks, depending upon the survival requirements. Up- and downregulation of protein expression could be detected at each developmental stage (unfed larvae/0M/2.5M/5M). To identify changes in tick protein expression, proteins were annotated using *Ixodes* database and changes in protein expression across different stages is shown via a heat map (figure 5A). Up- or downregulation of specific gene clusters point towards distinct changes owing to specific needs at each stage. Change in expression of top 50 gene clusters (figure 5B) shows major changes in tick metabolism upon moulting from larvae into nymphs (unfed to 0M) and upon starvation after moulting (0M to 2.5M), whereas most clusters remain unchanged from 10 weeks to 20 weeks of starvation (2.5M to 5M). Thereby, pointing towards major changes in ticks directly after moulting and upon entering starvation.

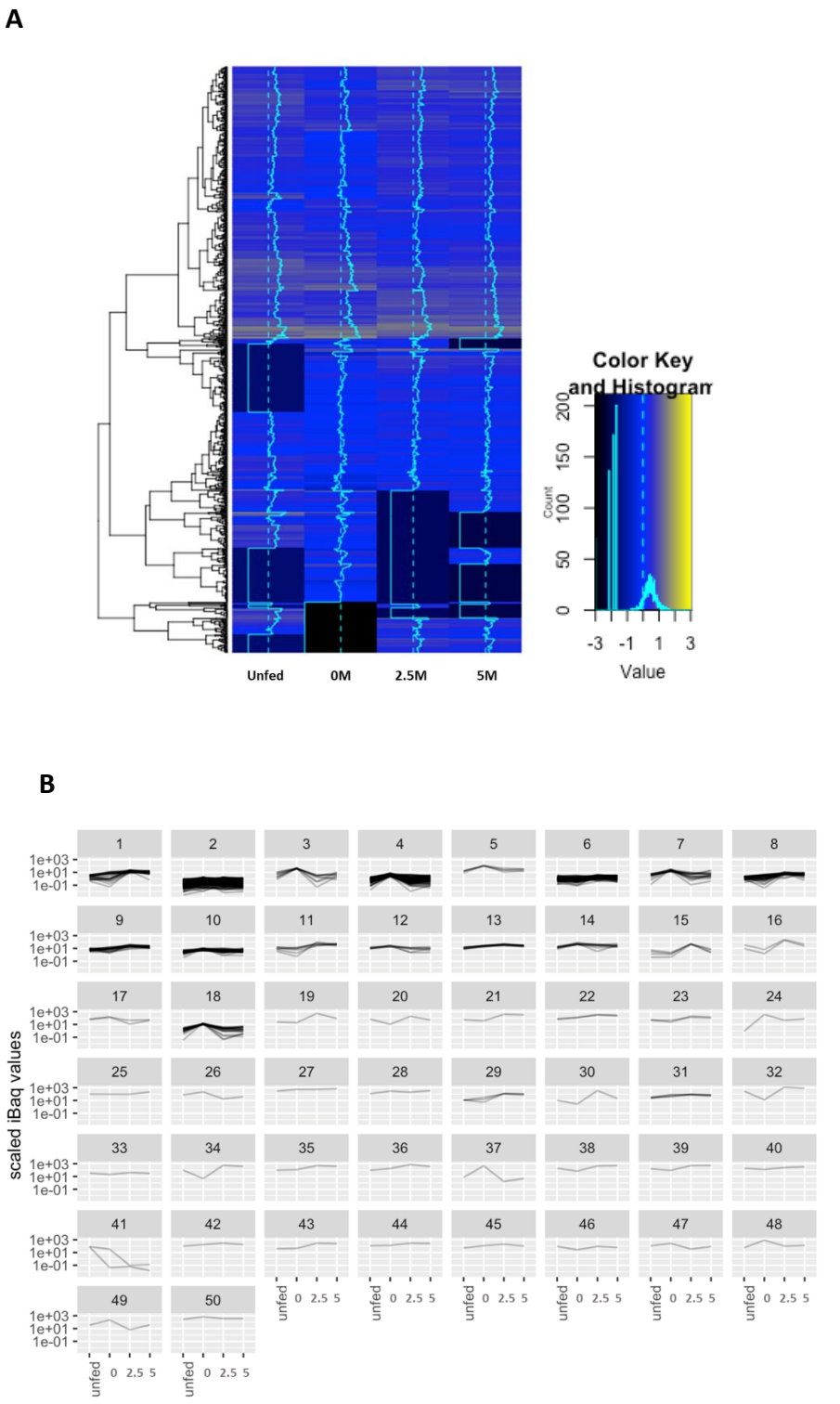

Figure 5. Differential tick protein expression in *Ixodes ricinus* ticks across different developmental stages: A. Heat map showing expression of different tick proteins (intensity) at larval stage (unfed) *versus* after moulting into nymphs (0M) and upon starvation upto 10 weeks (2.5M) and 20 weeks (5M). **B.** Clustering of the tick proteins over time based on scaled iBaq values shows several clusters with large changes in expression across different stages – unfed/0M/2.5M/5M.

DISCUSSION

Although the association of tick bites with sensitization to α-Gal is well established, the origin of α-Gal is still a matter of debate. The presence of α-Gal epitopes has been confirmed by immunoblotting and immunohistochemistry in several tick species (4, 6, 9, 10, 36), but so far it proved difficult to clearly identify the glycan carrying proteins. Tick proteins identified as α-Gal carrying glycoproteins include vitellogenins and α-2-macroglobulin protein groups (9), but a number of other tick proteins were identified as well as α-Gal carrying glycoproteins (4, 37). Due to the low abundance of material, all these findings rely solely on IgE-reactivity to protein spots isolated from 2D gels. A glycomic analysis of an isolated tick protein has not been described so far. In addition to an endogenous production of α-Gal, residual host blood could be a major source of α-Gal.

The objective of our study was to explore the stability of host mammalian protein over time, to identify these proteins and to assess their relative quantity. Tick larvae were fed on BALB/c mice under controlled conditions and protein content was analysed at 3 timepoints, including moulting into nymphs and a prolonged starvation period. No mouse protein was detected in unfed larvae, whereas a total of 21 mouse proteins were identified in nymphs, and 19 of these proteins were still detectable at the last time point i.e. 20 weeks after moulting. The most abundant proteins were serum albumin, hemoglobin, serine protease inhibitor 3k and serotransferrin. The peptide sequence coverage was particularly high for serum albumin and hemoglobin subunit alpha, pointing to the existence of proteins with intact N- and C-terminal ends. The normalization of the summed intensity of the detected mouse and tick peptides to the number of detectable peptides allowed a relative comparison of mouse and tick protein content. A surprisingly high amount of host protein was still detectable at 20 weeks of starvation.

The results of our study have implications for further research on the role of ticks in allergic sensitization, but also in a more general way regarding the evolution of the tick-host relationship and the immunological mechanisms to evade the host immune responses. As host protein is still detectable after moulting, all studies using unfed adult or nymph ticks to detect and confirm endogenous production of α-Gal need to be interpreted with caution. Of note, α-Gal was detected not only in the midgut and the whole body tick extract, but also in the salivary glands of several tick species (19, 25, 27). Regurgitation of gut content has been described in the soft tick *Ornithodorus moubata* (38). If all or part of these proteins originate from a previous host, this would mean that mammalian proteins are introduced into the next host upon feeding. When feeding on the same species, this likely would lead to some protection from the host immune responses, but when feeding on humans, protein epitopes as well as the α-Gal epitope, in conjunction with the immune modulatory molecules may lead to a Th2 response (39).

Neu5Gc, N-glycolylneuraminic acid, a sialic acid attached terminally to carbohydrate structures is another non-human monosaccharide for which findings are very similar to those of α-Gal. Sialylated structures were detected in *I. ricinus* salivary glands and midgut (40). Although ticks contain an active sialytransferase gene, metabolic labeling confirmed that the majority of the sialylated molecules in adult ticks originate from host blood (41). The passage of sialylated structures were mapped from the gut to the salivary glands (40). Metabolic recycling of glycans is a mechanism used by some pathogens, such as *Trypanosoma cruzi* for which transglycosylation by trans-sialidase has been demonstrated. The exact role of this conservation of carbohydrates and incorporation into parasite salivary proteins remain to be determined, but molecular mimicry is the most likely hypothesis as it would reduce immune responses in non-human hosts (27). A proteomic analysis of *I. scapularis* saliva at sequential feeding stages identified 582 tick and 83 rabbit proteins and it was hypothesized that host proteins

could be used to modulate interactions at the feeding site (42). Recently, a combination of tick and cattle host proteins were identified in the cement of *Rhipicephalus microplus*, an Ixodid tick. The content and function of the molecules varied with feeding and it was thought to play a role in attachment, sealing of the lesion and feeding, but also in the interaction and evasion from the host immune response (43).

Our study is the first analysing residual host blood proteins in *I. ricinus* ticks, a dominant tick species in Europe. The longitudinal design, paired with a high sensitivity proteomic analysis, allowed to relatively quantify mouse and tick protein content over time. A weakness of the study is certainly the lack of N-glycan analysis. However, as the content of α-Gal varies among different mammalian hosts, with mice being reported to have a low α-Gal content in blood (44, 45), a glycomic analysis would not have added more information. Moreover, for N-glycan analysis carbohydrates are cleaved-off of the respective glycoproteins and it is not traceable anymore whether they belonged to a tick or a host protein (27).

In conclusion, we showed that mouse proteins in larvae fed on mice survive moulting into nymphs and prolonged starvation. The most abundant proteins were serum albumin and hemoglobin alpha, for which a role in the elimination of excess iron has been postulated (42). In addition to endogenously produced α-Gal, we suggest that residual host protein is a plausible source of α-Gal whose content will depend on the previous host as well as on the duration of starvation.

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CHAPTER III

CHAPTER III. "α-Gal present on both glycolipids and glycoproteins contributes to immune response in meat-allergic patients"

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Author contributions:

"NC and CH were involved in all stages of the project, analyzed the data and wrote the manuscript. NC and KS conducted the experiments and analyzed the data; JF, FCM, MM and FH were the medical investigators recruiting patients and participating in data analysis and interpretation. CM performed the DLS analysis; DB did the statistical analysis; SB provided recombinant AP-N for antiserum production and participated in data interpretation; CBJ participated in the conceptualization of the digestion experiments. TB, MO and CH designed and supervised the project. All authors critically revised the manuscript and approved the final version."

PUBLISHED ARTICLE

DOI: https://doi.org/10.1016/j.jaci.2022.02.030

α-Gal present on both glycolipids and glycoproteins contributes to immune response in meat-allergic patients

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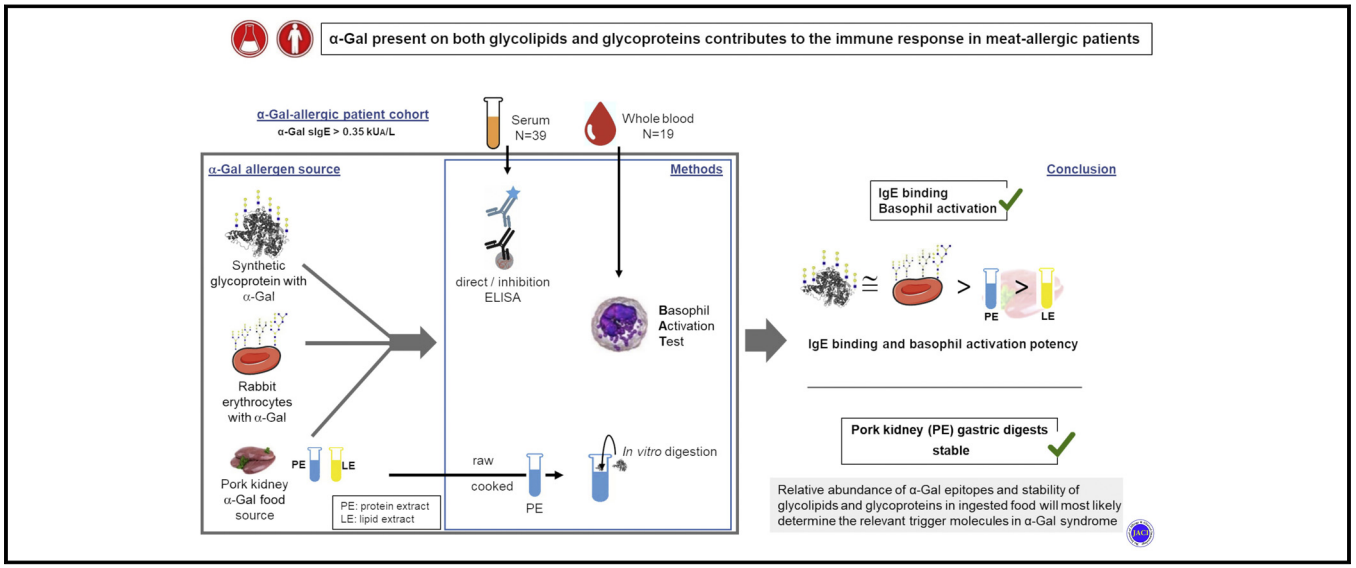
GRAPHICAL ABSTRACT



Background: The α-Gal syndrome is associated with the presence of IgE directed to the carbohydrate galactose-α-1,3-galactose (α-Gal) and is characterized by a delayed allergic reaction occurring 2 to 6 hours after ingestion of mammalian meat. On the basis of their slow digestion and processing kinetics, α-Gal–carrying glycolipids have been proposed as the main trigger of the delayed reaction.

Objective: We analyzed and compared the *in vitro* allergenicity of α-Gal–carrying glycoproteins and glycolipids from natural food sources.

Methods: Proteins and lipids were extracted from pork kidney (PK), beef, and chicken. Glycolipids were purified from rabbit erythrocytes. The presence of α-Gal and IgE binding of α-Gal–allergic patient sera (n = 39) was assessed by thin-layer chromatography as well as by direct and inhibition enzyme-linked immunosorbent assay. The *in vitro* allergenicity of glycoproteins and glycolipids from different meat extracts was determined by basophil activation test. Glycoprotein stability was evaluated by simulated gastric and intestinal digestion assays.

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Supported by bilateral funding from the Luxembourg National Research Fund (FNR), project C17/BM/11656090, and the Deutsche Forschungsgemeinschaft (DFG), projects FI2226/2-1, BI696/12-1, SFB1335 P17, and SFB1371 P06.

Disclosure of potential conflict of interest: The authors declare that they have no relevant conflicts of interest.

Received for publication August 24, 2021; revised February 10, 2022; accepted for publication February 21, 2022.

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0091-6749

https://doi.org/10.1016/j.jaci.2022.02.030

Results: α-Gal was detected on glycolipids of PK and beef. Patient IgE antibodies recognized α-Gal bound to glycoproteins and glycolipids, although binding to glycoproteins was more potent. Rabbit glycolipids were able to strongly activate patient basophils, whereas lipid extracts from PK and beef were also found to trigger basophil activation, but at a lower capacity compared to the respective protein extracts. Simulated gastric digestion assays of PK showed a high stability of α-Gal–carrying proteins in PK.

Conclusion: Both α-Gal–carrying glycoproteins and glycolipids are able to strongly activate patient basophils. In PK and beef, α-Gal epitopes seem to be less abundant on glycolipids than on glycoproteins, suggesting a major role of glycoproteins in delayed anaphylaxis upon consumption of these food sources. (J Allergy Clin Immunol 2022;■■■:■■■-■■■.)

Key words: *Glycolipids, α-Gal syndrome, red meat allergy, galactose-α-1,3-galactose, anaphylaxis, pork kidney, basophil activation, micelle formation,* in vitro *digestion*

Abbreviations used

α-Gal:	Galactose-α-1,3-galactose
AP-N:	Porcine aminopeptidase N
BAT:	Basophil activation test
EC_{50}:	Half-maximal effective concentration
ELISA:	Enzyme-linked immunosorbent assay
HSA:	Human serum albumin
IC_{50}:	Half-maximal inhibitory concentration
MALDI-TOF MS:	Matrix-assisted desorption ionization–time of flight mass spectrometry
PK:	Pork kidney
sIgE:	Specific IgE
TLC:	Thin-layer chromatography

The α-Gal syndrome is characterized by the presence of specific IgE (sIgE) antibodies directed at the disaccharide galactose-α-1,3-galactose (α-Gal).[1] Parenteral administration of α-Gal–carrying drugs such as cetuximab, a therapeutic chimeric mouse–human monoclonal antibody, or bovine-derived gelatin in volume colloids or vaccines, leads to rapid onset of symptoms in α-Gal–allergic patients. In contrast, oral uptake of mammalian meat and innards, or dairy products is characterized by a delayed onset of symptoms of up to 2 to 6 hours.[2] This time delay may be shortened by certain foods that are particularly rich in α-Gal (eg, pork innards)[3,4] or by exogenous factors with an impact on digestion, such as alcohol, physical exercise, or nonsteroidal anti-inflammatory drugs.[2] These findings strongly support the assumption that the delay of symptom onset upon ingestion is related to the digestion process and not to the carbohydrate epitope. The α-Gal epitope is also present on glycolipids.[5] Because digestion and absorption of glycolipids are much slower than the digestion of glycoproteins, it has been hypothesized that α-Gal–carrying glycolipids are responsible for the delayed onset of symptoms upon ingestion of mammalian meat.[6] Román-Carrasco et al[7] used an *in vitro* model of Caco-2 cells to show that α-Gal bound to lipids was able to cross the cell monolayer, whereas α-Gal bound to proteins was not detectable on the basal side of the cell layer. The first evidence that sIgE of patients with α-Gal allergy is able to recognize the α-Gal epitope present on a lipid backbone comes from a study by Iweala et al[8] in which patient sIgE recognized the α-Gal epitope on receipt of isoglobotrihexosylceramide (iGb3) as well as a synthetic iGb3 analog, PBS-113. Donor basophils could be activated using patient blood and PBS-113, thus demonstrating that the α-Gal epitope bound to a synthetic lipid is able to activate basophils via an IgE-mediated mechanism.

In the present study, we analyzed recognition of the α-Gal epitope on natural mammalian glycoproteins and glycolipids by sIgE of patients with α-Gal syndrome. We compared the *in vitro* allergenicity of glycoprotein and glycolipid preparations obtained from different mammalian sources, exploring the rationale for delayed symptoms upon ingestion of mammalian meat. Our data show that both glycolipids and glycoproteins are allergenic *in vitro*. They also suggest that as a result of the high stability of α-Gal–carrying glycoproteins during gastric digestion, proteins may also contribute to delayed anaphylaxis upon ingestion of pork kidney (PK).

METHODS

Patients and sera

Symptomatic α-Gal–allergic patients were recruited at the outpatient clinic of the Allergology Unit of the Department of Dermatology of Eberhard Karls University in Tübingen (n = 14) and at the National Immunology–Allergology Unit at the Centre Hospitalier de Luxembourg (n = 19). In addition, 6 patients with suspected meat allergy and confirmed type I sensitization to α-Gal were included from both clinics. Patients were included on the basis of a positive clinical history and/or suspected meat allergy and an sIgE titer to α-Gal of >0.35 kU_A/L (mean sIgE, 22.20 kU_A/L; range, 0.49 to >100 kU_A/L) (ImmunoCAP; Thermo Fisher Scientific, Uppsala, Sweden) (see Table E1 in this article's Online Repository at www.jacionline.org). Milk, pork, and beef (kidney, meat) were tested by prick to prick and gelatin (Gelfundin 4%) by intracutaneous application. Thirty-eight percent of subjects were female, and mean age was 47 years (range, 13-84 years).

The study was approved by the national committee for medical research ethics in Luxembourg (201605/03 and 201910/04) and by the ethics commission of the University Medical Faculty in Tübingen (158/2016BO1). Written informed consent was obtained from all study participants. Control and serum pool details are provided in the Methods section in the Online Repository.

Extraction and analysis of glycolipids and lipids

Glycolipids were extracted from rabbit erythrocytes (Innovative Research, Novi, Mich) as described by Galili et al[5] with some modifications. Because they were highly enriched in α-Gal–carrying glycolipids, they served as a model and control for comparison between PK and beef lipids. Total lipids were extracted from PK, Irish beef fillet, and chicken fillet meat as described by Román-Carrasco et al.[7] Extracted rabbit glycolipids and meat lipids were analyzed by thin-layer chromatography (TLC). Glycolipids extracted from rabbit erythrocytes and PK were analyzed by Asparia Glycomics (San Sebastián, Spain) as per their established protocol.[9] Details are provided in the Methods section in the Online Repository.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis and immunoblot

Protein extracts from PK and meat were separated under reducing conditions on a 7.5% sodium dodecyl sulfate–polyacrylamide gel electrophoresis gel and immunoblotted as described elsewhere.[10] Proteins carrying the carbohydrate α-Gal were visualized using mouse monoclonal anti–α-Gal IgM (M86) antibody (Enzo Life Sciences, Lausen, Switzerland). Porcine aminopeptidase N (AP-N) was detected using polyclonal serum obtained

from rabbits immunized with recombinant AP-N produced in HEK cells (see the Methods section in the Online Repository).

Detection of sIgE by enzyme-linked immunosorbent assay (ELISA) and relative quantification of α-Gal epitopes by inhibition ELISA

sIgE to α-Gal was detected as described with some modifications,[10] as indicated in the Methods section in the Online Repository.

The relative quantification of α-Gal epitopes was assessed by inhibition ELISA. Microtiter plates were coated overnight with 2 μg/mL of α-Gal human serum albumin (HSA). PK and beef protein and lipid extracts, rabbit glycolipids, and α-Gal HSA were diluted in phosphate-buffered saline to reach a concentration of 200/20/2/0.2/0.02 μg/mL and incubated 1:1 (vol/vol) with an α-Gal–allergic patient serum pool (diluted 1:20 in phosphate-buffered saline) for 2 hours at room temperature. Inhibited samples were added to the microplate wells and incubated overnight at 4°C. Bound IgE was detected the next day. A description is provided in the Methods section in the Online Repository.

Basophil activation test

The Flow CAST (Bühlmann Laboratories, Schönenbuch, Switzerland) assay was used for quantitative measurement of *in vitro* basophil activation. Venous blood was collected from 19 patients; the assay was performed within 24 hours using increasing concentrations of allergen extracts, as previously described.[11]

Simulated gastric and intestinal digestion

Simulated gastric and intestinal fluid were prepared as described elsewhere.[12] Details are provided in the Methods section in the Online Repository. Before gastric digestion, all components were heated to 37°C, mixed, and the pH adjusted to 3. Thereafter, 0.5 mL of soluble protein extract or PK whole tissue extract (raw or cooked) was added 1:1 (vol/vol); the resultant digestion mixture had a pepsin activity of 2000 U/mL. For intestinal digestion, pancreatin in simulated intestinal fluid was mixed with an equal volume of gastric chyme; the final mixture had a trypsin activity of 100 U/mL and a concentration of 10 mmol/L bile acids. Simulated digests were incubated at 37°C and aliquots formed after the indicated time intervals.

RESULTS

Glycolipids isolated from rabbit erythrocytes are recognized by IgE antibodies of α-Gal–allergic patients

To investigate binding of IgE antibodies from α-Gal–allergic patients to the α-Gal epitope carried by a natural glycolipid molecule, glycolipids were extracted from rabbit erythrocytes. Rabbit erythrocytes have previously been shown to carry high amounts of α-Gal epitope.[5] IgE binding to glycolipids was compared to IgE binding to α-Gal HSA (a commercial glycoprotein of α-Gal coupled to HSA).

Sera from patients with α-Gal syndrome were analyzed for reactivity against glycolipids by ELISA (Fig 1, *A*). α-Gal bound to glycolipids was recognized by patient IgE; however, IgE binding levels to α-Gal HSA were significantly higher ($P < .0001$). The monoclonal antibody M86, which has been shown to recognize the terminal disaccharide, the α-Gal epitope,[13] has been used as control. Different dilutions of M86 showed no significant difference in the recognition of the α-Gal epitope on both molecular backbones (Wilcoxon matched-pair signed-rank test, $P = .062$) (Fig 1, *B*). IgE binding to α-Gal HSA and rabbit glycolipids is strongly correlated ($P < .0001$), and it also correlates with sIgE levels measured by ImmunoCAP ($P < .0001$) (Fig 1, *C*). The α-Gal epitope present on glycoproteins and glycolipids is well recognized by sIgE from patients and by the murine monoclonal anti–α-Gal IgM antibody M86.

Terminal α-Gal epitopes are present in high quantity on rabbit glycolipids

For further characterization, the rabbit erythrocyte extract was separated on a silica gel plate by TLC. Glycans and lipids were stained to visualize glycolipids on the TLC-resolved plate; the presence of carbohydrates (glycans) and lipids was confirmed by orcinol and primulin staining, respectively. A mix of 3 commercially available glycolipids, used as reference, migrated over a longer distance than the rabbit erythrocyte extract, suggesting the presence of more complex carbohydrate structures in rabbit glycolipids (Fig 2, *A*).

Next, the presence of α-Gal epitopes on glycolipids was confirmed by TLC immunoblotting using the anti–α-Gal IgM antibody M86, which detected multiple distinct bands corresponding to the carbohydrate bands previously observed with orcinol staining, thereby confirming presence of α-Gal on most of the glycolipids in the extract. Moreover, compared to the 2 reference α-Gal glycolipids, most of α-Gal–carrying rabbit glycolipids were closer to the baseline and spread over a large migration range, thus confirming the presence of mostly complex α-Gal–carrying glycolipids in the extract (Fig 2, *A*). Because rabbit glycolipids are a highly enriched fraction of α-Gal–carrying glycolipids, they will serve as a reference for all subsequent analyses and are hereafter referred to as rabbit glycolipids.

PK has been shown to be a potent trigger of severe anaphylactic reactions to red meat.[4] Because sIgE binds to glycolipids and glycoproteins carrying α-Gal, the allergenic potentials of both α-Gal–carrying molecules from PK and beef were analyzed. First, lipid extracts from PK, beef, and chicken were resolved on a silica gel plate by TLC, then stained with orcinol to visualize carbohydrates. Orcinol staining showed a higher abundance of carbohydrates in beef and chicken lipid extract compared to PK lipid extract (Fig 2, *B*). Second, the α-Gal epitope was detected in the TLC immunoblot using a patient serum pool. In the PK lipid extract, patient IgE revealed 2 bands, one corresponding to glycolipids carrying short carbohydrate chains and the other at baseline, whereas a major and a minor band were observed in the beef lipid extract (Fig 2, *B*). No α-Gal was detected in chicken lipid, as expected. No signal was detected in PK and beef extract using the M86 antibody (data not shown). However, in inhibition ELISA, PK lipids could inhibit M86 at 100 μg/mL (51% inhibition) whereas no inhibition was observed with the beef lipids (see Fig E1 in the Online Repository at www.jacionline.org), thus suggesting a lower sensitivity of M86 antibody compared to the patient pool.

In addition, the glycan structures on glycolipids and their relative quantity in the extract were determined by glycomic analysis (Fig 3). In rabbit erythrocyte extract, the presence of numerous glycans with terminal α-Gal epitopes was confirmed by matrix-assisted desorption ionization–time of flight mass spectrometry (MALDI-TOF MS/MS) (see Fig E2 in the Online Repository at www.jacionline.org). Relative quantification of glycans via ultra performance liquid chromatography coupled to fluorescence detector–based fluorescence detection showed biantennary glycans with 2 terminal Gal-α-1,3-Gal residues to be present in a relatively large quantity (50% of the total glycans

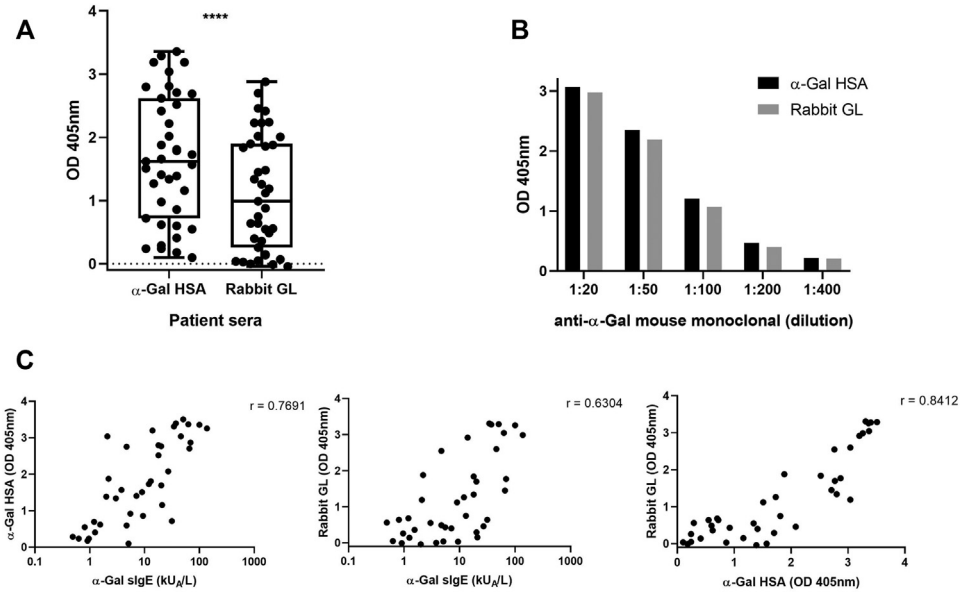

FIG 1. Glycolipids from rabbit erythrocytes recognized by IgE from α-Gal–allergic patient sera and by an anti–α-Gal antibody. (**A**) IgE reactivity of patient sera (n = 39; sIgE = 0.49-100 kU_A/L) against rabbit glycolipids (GL) and α-Gal HSA determined in ELISA. Box plot of optical density (OD) values with median; Wilcoxon matched-pair signed-rank test ($P < .0001$). OD values of negative control subtracted. (**B**) Murine monoclonal anti–α-Gal IgM (M86) binds α-Gal on both α-Gal HSA and rabbit GL; Wilcoxon matched-pair signed-rank test ($P = .0625$). (**C**) Spearman correlation between sIgE level and OD values observed in ELISA (α-Gal HSA, $r = 0.7691$; rabbit glycolipids, $r = 0.6304$), and α-Gal HSA OD vs rabbit glycolipids OD values ($r = 0.8412$).

detected), followed by triantennary structures with 2 α-Gal epitopes (10%) (Fig 3).[14] Glycan linkages were determined via MALDI-TOF MS/MS analysis (representative spectra are shown in Fig E3 in the Online Repository). Glycomic analysis of the PK lipid extract (see Fig E4 in the Online Repository) was not able to identify glycans or to quantify them relatively as a result of their low abundance in the lipid extract. Because of the low abundance of α-Gal epitopes on beef glycolipids, glycomic analysis was not attempted.

α-Gal–carrying glycans are more abundant on glycoproteins than on glycolipids

Extracted lipids were coated onto ELISA plates with a hydrophobic surface and probed with either patient serum pool or monoclonal antibody M86. However, as a result of the low abundance of α-Gal–carrying glycolipids, no signal was observed with patient sera or M86 antibody (data not shown). We therefore quantified the presence of α-Gal epitopes by inhibition immunoassay.

IgE binding of an α-Gal–allergic patient serum pool to α-Gal HSA was inhibited by the addition of PK, beef and chicken, and protein and lipid extracts at varying concentrations (Fig 4), and half-maximal inhibitory concentration (IC_{50}) value was calculated by nonlinear regression with a 3-parameter analysis. Inhibition with the model glycoprotein α-Gal HSA and rabbit glycolipids produced IC_{50} values of 0.22 μg/mL and 0.30 μg/mL, respectively; an IC_{50} value of 3.90 μg/mL for PK protein extract; and an IC_{50} for PK lipid extract of 50.64 μg/mL. IC_{50} values could not be calculated for beef protein and lipids because inhibition reached merely 50% and 24%, respectively, at highest concentration of the inhibitor. The inhibition assay resulted in the following ranking with regard to the abundance of α-Gal epitopes: α-Gal HSA > rabbit erythrocyte glycolipids > PK glycoproteins > PK glycolipids > beef glycoproteins > beef glycolipids.

Protein extracts from meat have a higher *in vitro* allergenicity than lipid extracts

The allergenic potential of α-Gal–carrying glycolipids and glycoproteins was analyzed by basophil activation tests (BATs) in 19 α-Gal–allergic patients. Three patients were excluded because they had nonresponsive disease, and 3 were excluded as a result of high background activation (range, 32-42%). Increasing doses of α-Gal HSA, rabbit glycolipids, and meat/PK protein and lipid extracts, ranging from 0.1 μg/mL to 100 μg/mL, were added to patients' whole blood, and basophils were analyzed by flow cytometry. The fluorescence-activated cell sorting gating strategy as well as activation plots from a representative patient sample are shown in Fig E5 in the Online Repository at www.jacionline.org.

Rabbit glycolipids strongly activated basophils and reached maximum activation at 1 μg/mL, whereas α-Gal HSA reached the maximum at 100 ng/mL (Fig 5, *A*). Patient basophils were less reactive to PK protein and lipid extracts, and concentrations above 10 μg/mL were needed to initiate a significant activation (Fig 5, *B*). Responses to beef protein and lipid extract were very low, and were not detectable for chicken lipid extract (Fig 5, *C,* and see Fig E6, *A,* in the Online Repository at www.jacionline.org). No activation was observed in allergic and healthy controls (Fig E6, *B*).

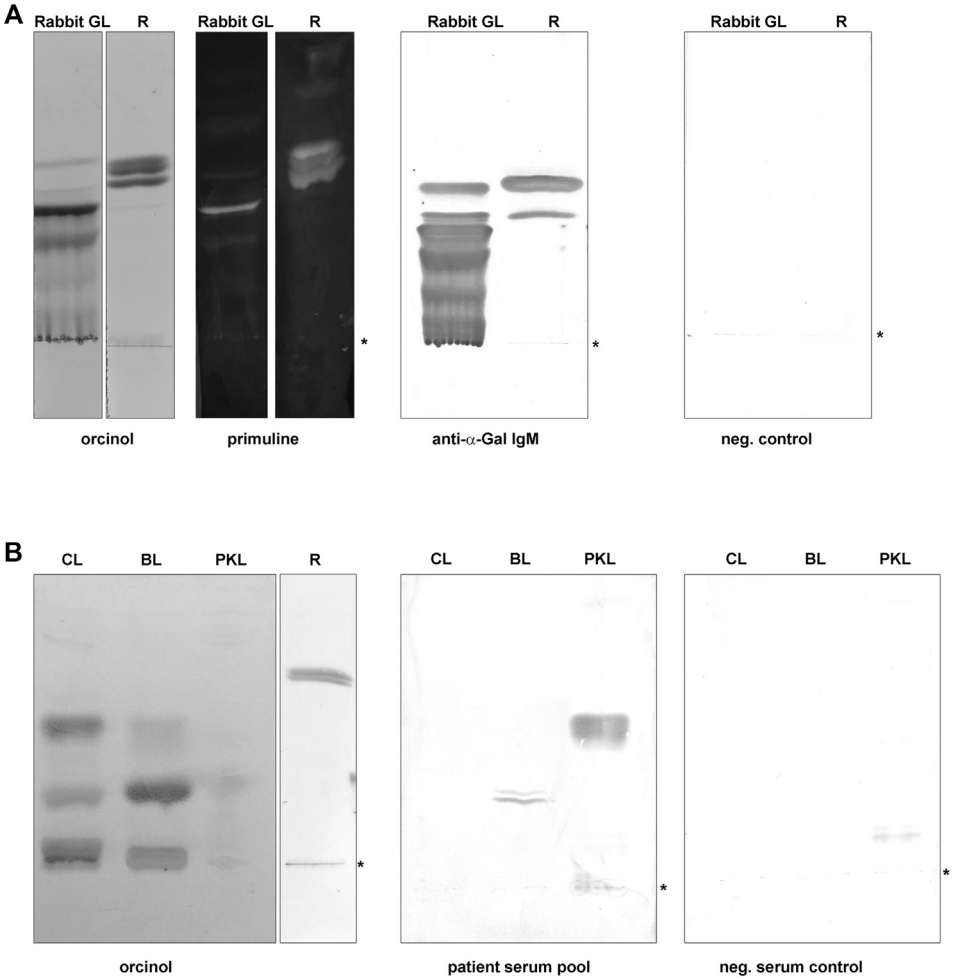

FIG 2. α-Gal on glycolipids recognized by anti–α-Gal antibodies. (**A**) TLC showing carbohydrates (orcinol staining) and lipids (primulin staining) in rabbit erythrocyte extract and reference glycolipids. TLC immunostaining of rabbit glycolipids with the murine monoclonal anti–α-Gal IgM (M86). R indicates mix of reference glycolipids Galα-1,3-Galβ1-HDPE, Galβ-1,4-GlcNAcβ1-HDPE, and Galα-1,3-Galβ-1,4-GlcNAcα1-HDPE. (**B**) Carbohydrate staining (orcinol) of lipid extracts from chicken (*CL*), beef (*BL*), and PK (*PKL*), and reference glycolipids (*R*). TLC immunostaining of CL, BL, and PKL with an α-Gal–allergic patient serum pool (n = 21; sIgE α-Gal = 28.1 kU_A/L); a serum without sIgE to α-Gal was used as negative control. *Sample spotting point.

On the basis of the half-maximal effective concentration (EC_{50}), we established the following ranking: α-Gal HSA (0.009 μg/mL) > rabbit glycolipids (0.059 μg/mL) > PK protein (6.806 μg/mL) > PK lipids (154.474 μg/mL). All curve comparisons reached statistical significance, with the exception of the pair α-Gal HSA/rabbit glycolipids. EC_{50} could not be determined for beef protein and lipids because basophil activation was very low. This ranking also reflects the abundance of α-Gal epitopes quantified by inhibition ELISA (Fig 4). Whereas α-Gal HSA and rabbit glycolipids showed similar epitope abundance in the ELISA assay, EC_{50} of α-Gal HSA was 6-fold lower in the BAT assay; however, it was not statistically significant. The area under curve gave the following ranking: rabbit glycolipids (3.942) > α-Gal HSA (2.977) > PK proteins (1.536) > PK lipids (0.895). PK and beef protein extracts were globally more allergenic than the corresponding lipid extracts (Fig 5).

Lipids form micelles, as confirmed by dynamic light scattering

Rabbit glycolipids were able to induce a strong basophil activation, implying the presence of at least 2 epitopes per molecule. Biantennary glycans with 2 terminal α-Gal epitopes were present on the majority of the glycolipids; however, the formation of micelles would enable multimerization of epitopes and enhance allergenicity. The formation of micelles by lipids and glycolipids from meat, PK, and rabbit erythrocytes extract was analyzed by dynamic light scattering.

Rabbit glycolipids formed micelles with a diameter of 500 nm from approximately 76% of the population. Trimodal distribution was observed, with the diameter varying from 30 to 1000 nm and a polydispersity index of 0.65. The largest population exhibited a diameter close to 800 nm. A bimodal distribution was observed for the PK (diameter range 50-550 nm, 80% of micelles) and beef lipid (diameter range 140-400 nm, 90% of micelles) samples with

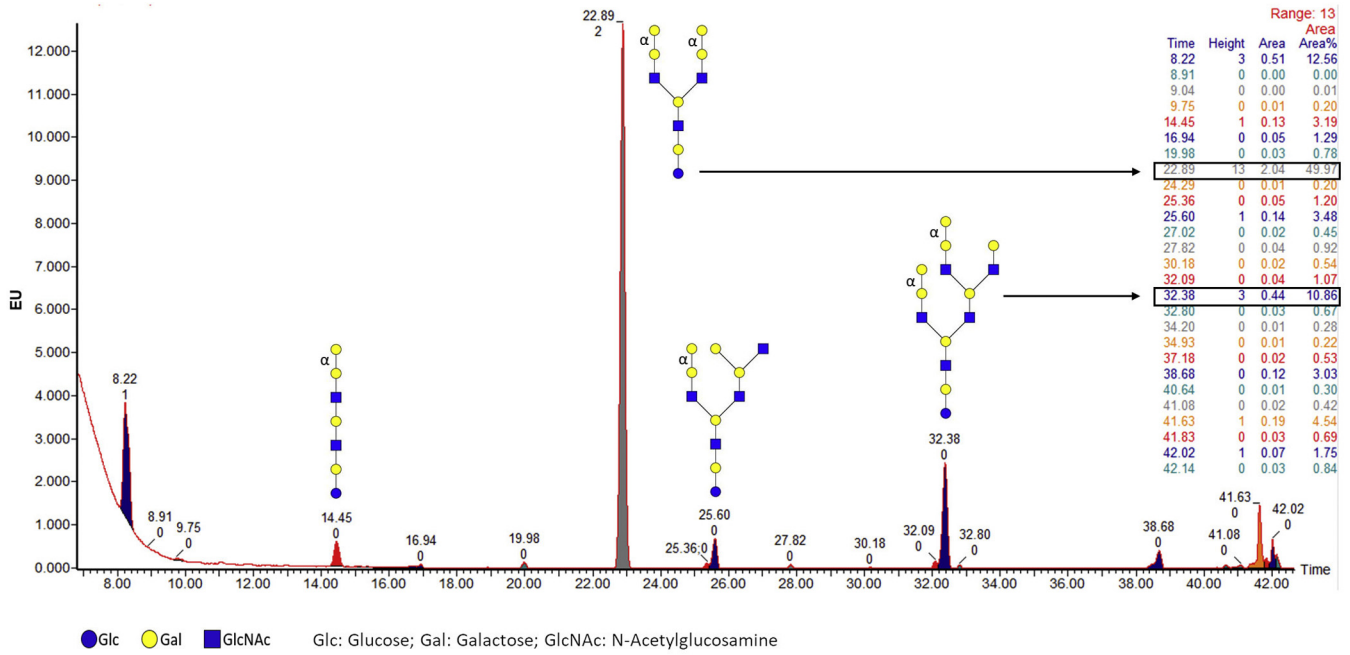

FIG 3. Numerous glycans with terminal α-Gal epitope, also referred to as rabbit glycolipids (*GL*), were detected in rabbit erythrocyte extract. Relative quantification of α-Gal glycolipids via ultra performance liquid chromatography coupled to fluorescence detector. Glycan nomenclature as per SNFG (Symbol Nomenclature for Glycans) guidelines.[14]

an average diameter of 300 nm and 270 nm, and a polydispersity index corresponding to 0.38 and 0.31, respectively (see Fig E7 in the Online Repository at www.jacionline.org). Chicken lipids (diameter range 190-450 nm, 80% of micelles) formed micelles with an average size of 300 nm and a polydispersity index of 0.35. The formation of micelles was confirmed for all preparations of lipids and glycolipids, with rabbit glycolipids forming the largest micelles, thereby confirming the possibility of multivalent glycan epitopes on the surface of micelles enabling them to cross-link IgE and activate basophils.

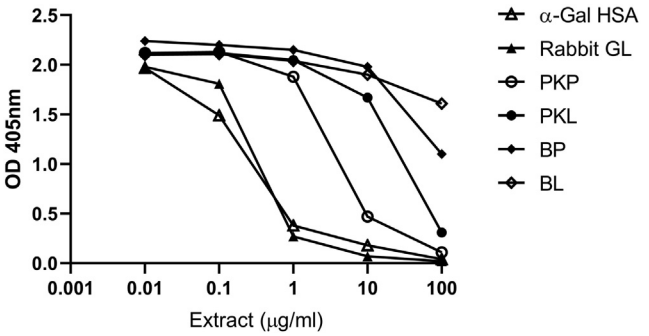

FIG 4. α-Gal–carrying molecules are present in a higher quantity in PK (**A**) and beef (**B**) protein extract than lipid extract. For inhibition ELISA, α-Gal HSA (2 μg/mL) precoated to a microtiter plate was detected by patient IgE (serum pool, sIgE = 74.8 kU_A/L; diluted 1:20) preinhibited with PK (*PKL,* lipid; *PKP,* protein) or beef (*BL,* lipid; *BP,* protein) meat extracts, α-Gal HSA, or rabbit glycolipids (GL) at increasing concentrations (0.01-100 μg/mL).

α-Gal–carrying glycoproteins and aminopeptidase N are stable under gastrointestinal digestion conditions

Because protein extracts from PK were found to be abundant in α-Gal epitopes (Fig 4) and to be highly reactive in the BAT assay (Fig 5), we investigated stability of α-Gal–carrying glycoproteins in simulated gastric and intestinal digestion assays (Fig 6).

The soluble fraction of PK protein extract was subjected to gastric digestion and analyzed at sequential time points for the presence of α-Gal–carrying glycoproteins and for AP-N, an abundant PK protein previously confirmed to induce basophil activation in α-Gal–allergic patients.[3] α-Gal reactive protein bands and AP-N were found to be stable during gastric digestion for up to 2 hours, while the overall protein profile tends to degrade and small peptides accumulate over time (Fig 6). Upon sequential gastric and intestinal digestion, α-Gal–carrying proteins begin to degrade; the size of α-Gal peptides decreased to 40 kDa, whereas AP-N was still detectable at high molecular weight after 2 hours (Fig 6).

To investigate gastric and intestinal digestion under more physiological conditions, complete raw and cooked PK protein extract including any insoluble material was used in a sequential digestion assay (see Fig E8 in the Online Repository at www.jacionline.org). In raw PK extract, α-Gal–carrying glycoproteins including AP-N were highly stable during gastric and intestinal digestion (Fig E8, *lanes 1-4*). In cooked PK, glycoproteins survived gastric digestion but degraded during the intestinal phase (Fig E8, *lanes 5-8*). The weak signal of AP-N in cooked PK may be due to a change in protein conformation upon heating, paired with a loss of antibody recognition. α-Gal epitopes are not affected by heating but become undetectable upon prolonged

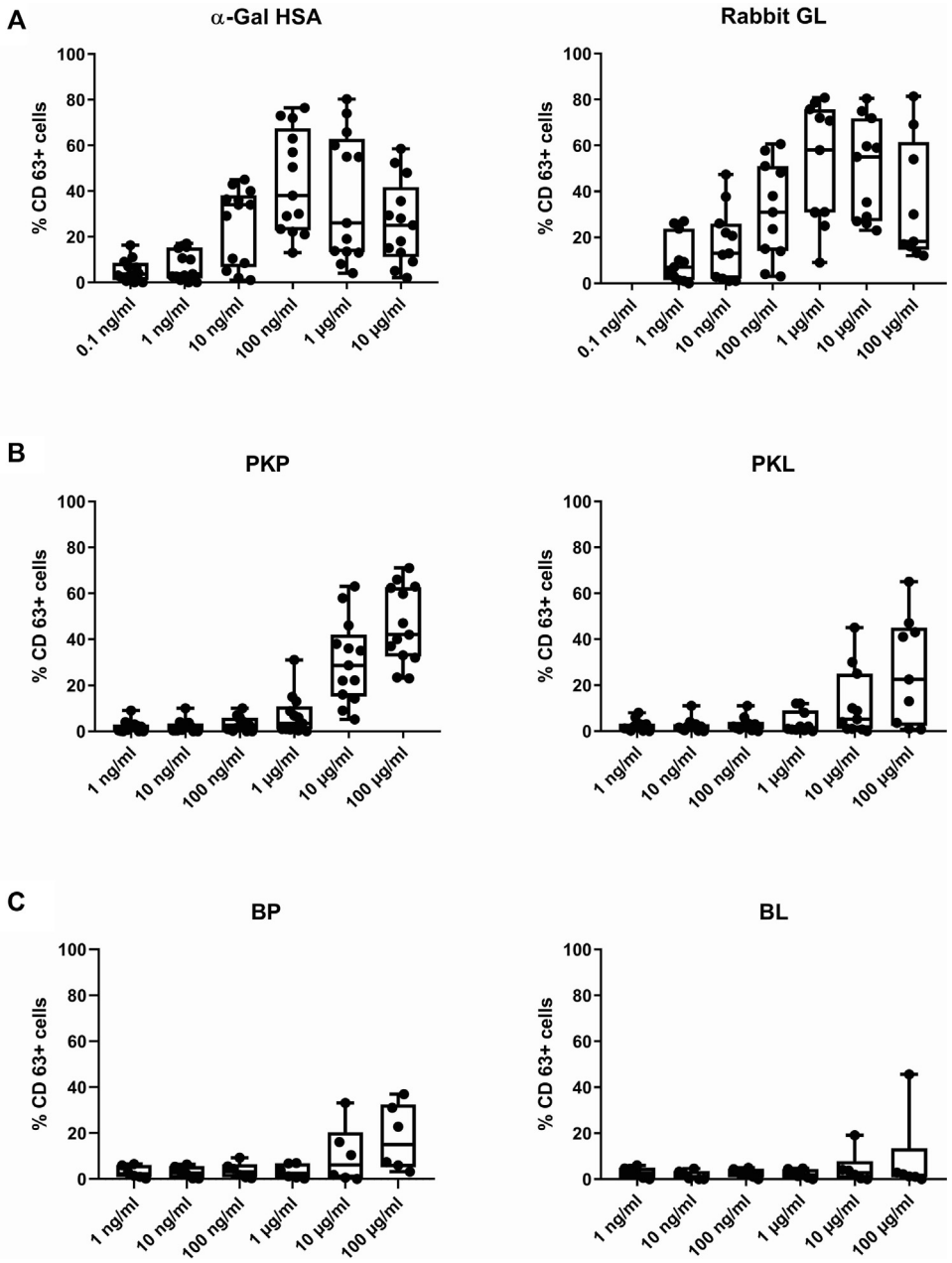

FIG 5. Basophils from clinically reactive α-Gal–allergic patients (sIgE 0.74-50.8 kU_A/L) are activated on stimulation with both α-Gal–carrying glycoproteins and glycolipids. Box plot with whiskers (minimum to maximum) showing percentage of CD63 upregulation with increasing dose of extracts in BAT assay. (**A**) α-Gal HSA (glycoprotein; n = 13) and rabbit glycolipids (*GL;* n = 11). (**B**) PK protein (*PKP;* n = 13) and PK lipid (*PKL;* n = 11) extracts. (**C**) Beef protein (*BP*) and beef lipid (*BL*) extracts (n = 6).

intestinal digestion. These findings suggest that cooked PK might be less resistant to intestinal digestion.

DISCUSSION

In food allergy, symptoms mostly arise within minutes after ingestion of the allergenic food source,[15] whereas patients with α-Gal syndrome usually experience a delay of more than 2 hours after ingestion of mammalian food products.[16] A majority of patients experience urticaria and gastrointestinal symptoms, followed by angioedema and anaphylaxis,[16] suggesting an important role of both mast cells and basophils. Although several α-Gal–carrying glycoproteins have been identified in PK, beef, and milk,[3,17-19] a currently favored hypothesis is that glycolipids are the major trigger of symptoms because the kinetics of their digestion process correlates with the appearance of symptoms.[20] This hypothesis is supported by a recent study by Román-Carrasco et al[7] showing that α-Gal bound to lipids was able to cross the cell monolayer and was packaged into chylomicrons.

In the present study, we recruited a group of patients with α-Gal syndrome to analyze sIgE binding and the *in vitro* allergenicity of naturally occurring glycolipids and glycoproteins from

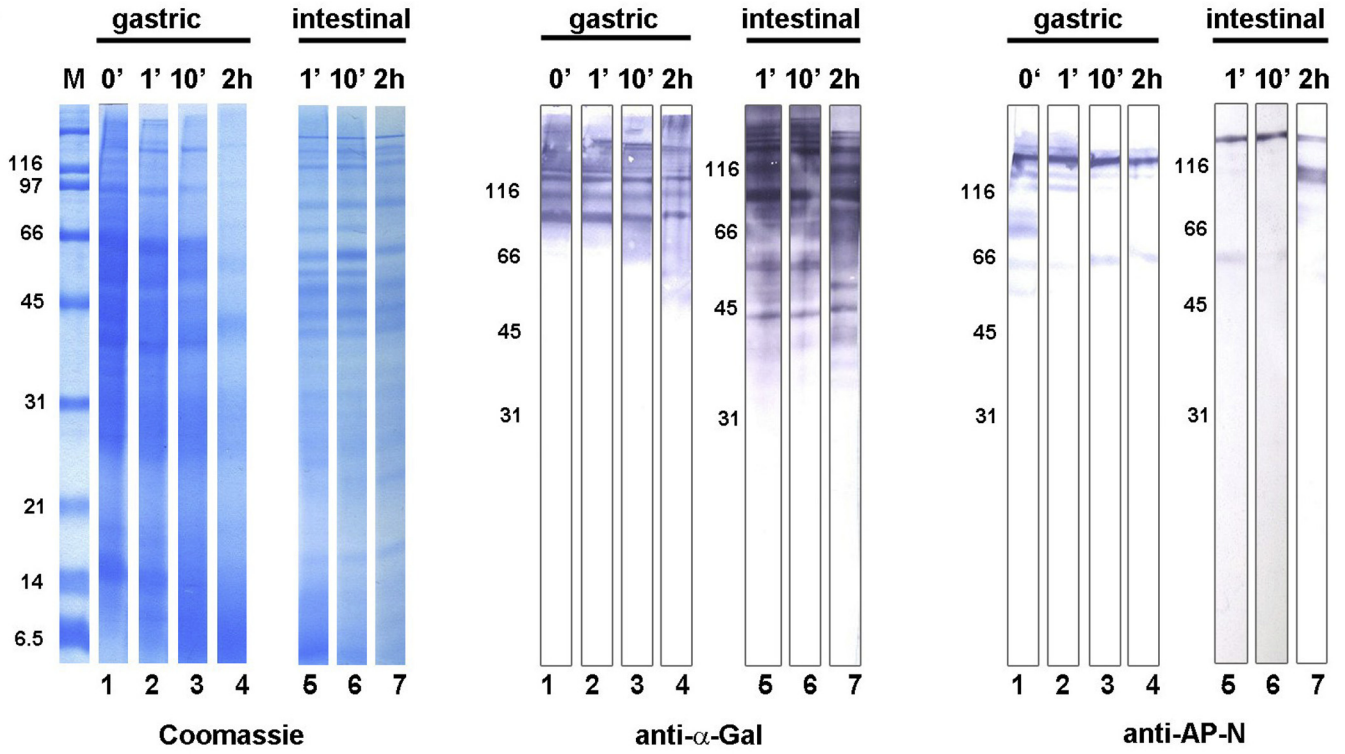

FIG 6. α-Gal–carrying glycoproteins in PK are highly stable under gastric digestion conditions. PK-soluble protein extract–Coomassie gel and immunoblot of simulated gastric and intestinal digest over a period of 0 minutes to 2 hours. Detection with anti–α-Gal and anti–AP-N antibodies in immunoblot. Time point of sample analysis indicated above each lane.

mammalian sources. Rabbit glycolipids were already known to carry multiple α-Gal epitopes and to be able to bind human IgG directed to α-Gal.[5,21] We therefore extracted glycolipids from rabbit erythrocytes according to an established protocol and used them as reference glycolipids to study and compare IgE binding of sera from patients with α-Gal syndrome to different lipid and protein meat extracts.

Rabbit glycolipids showed very high IgE binding and strong basophil activation comparable to that of α-Gal HSA, to which multiple α-Gal epitopes have been covalently linked. Glycomic analysis of rabbit glycolipids confirmed the presence of multiple carbohydrate structures carrying α-Gal, with the most abundant one accounting for 50% of the total carbohydrate load as biantennary structures with 2 terminal α-Gal epitopes. In contrast, α-Gal–carrying glycolipids were undetectable by glycomic analysis of lipids extracted from PK. The presence of α-Gal could be confirmed by TLC immunostaining, however. These findings can be explained by the lower sensitivity of the glycomic analysis when using whole lipid extracts. Previous studies using prefractionation of glycolipids have succeeded in detecting terminal α-Gal in different porcine organs, and kidney was found to contain the highest amounts of Gal-α1,3–terminated glycolipids.[22,23]

After having established IgE binding and *in vitro* allergenicity of rabbit glycolipids, we analyzed proteins and lipids from PK and beef, 2 major meat sources triggering α-Gal–related symptoms. Although α-Gal epitopes could be detected by TLC immunostaining of beef and PK lipids, their abundance was low, and they were undetectable by direct IgE ELISA using patient sera or a monoclonal antibody. The relative abundance of α-Gal epitopes could be determined by inhibition ELISA, however, and the following ranking could be established: PK protein extract contained more α-Gal epitopes than PK lipid extract, and beef protein extract also contained more α-Gal than beef lipid extract. The ranking in IgE binding was mirrored in basophil activation assays where protein extracts triggered stronger responses than lipids extracts, and PK was more potent than beef. These are in line with previous findings that PK is more abundant in α-Gal–carrying glycoproteins and glycolipids than other porcine tissues.[22,24,25] They also correlate well with clinical data showing that PK is the most potent trigger of α-Gal–related anaphylaxis.[3,4,26] The respective abundance of α-Gal–carrying glycoproteins and glycolipids may of course vary for different pieces of meat, depending on their origin and fat content.

The concentrations of PK protein extract needed for significant stimulation of basophils are 10-100 μg/mL. These doses are about 10 times higher compared to BAT assays with IgE directed at protein epitopes,[27] but this is well in line with our previously determined doses[3] and doses recently reported for α-Gal–carrying milk proteins, where maximal activation was reached at 10 and 100-200 μg/mL for single milk allergens,[18] most likely reflecting the respective abundance of α-Gal epitopes in these allergen sources. In contrast, maximal activation was achieved by α-Gal HSA and rabbit glycolipids at 100 ng/mL and 1 μg/mL, respectively, demonstrating that a strong basophil activation can be obtained *in vitro* by α-Gal irrespective of the biological carrier molecule. The lower activation by beef and PK glycolipids is most likely due to the relatively low abundance of α-Gal epitopes in the lipid extracts. As a result of their hydrophobic nature, rabbit

glycolipids, and PK and beef lipids formed micelles in aqueous conditions in *in vitro* experiments, exposing α-Gal on the surface of these structures. The generation of large structures with multiple epitopes may enhance their *in vitro* allergenicity.[28] It is to be expected that *in vivo* packaging into chylomicrons formed during absorption of fat in the ileum will also expose multiple α-Gal epitopes on the surface. The size of chylomicrons in humans is in a similar range as to what we found for micelles (200-1000 nm).[29] Further conversion into low- and high-density lipoproteins will decrease particle size to 7 to 30 nm, thus enabling tissue penetration and mast cell activation.[6,29]

Because protein extracts are more allergenic *in vitro* than the respective lipid extracts, we investigated their stability on gastric and intestinal digestion. Simulated gastric digestion revealed a high stability of proteins carrying α-Gal epitopes. Similarly, AP-N, a PK protein previously found to be an important carrier of α-Gal in PK,[3] was found to be stable for 2 hours of gastric digestion, implying that α-Gal would reach the intestine bound to high-molecular-weight proteins and that the carbohydrate would only become bioavailable after breaking down into smaller peptides during intestinal digestion, correlating with a delayed appearance of symptoms. A previous study reported that the *ex vivo* basophil activation during a food challenge occurred within the same time frame as clinical symptoms, providing clear evidence of an IgE-mediated activation.[30] This finding is further confirmed by the recent work of Eller et al[31] showing that a blended PK smoothie that is thought to have a shortened transition time in the stomach was able to significantly shorten the time of absorption of α-Gal into the bloodstream. Another study using gastric digestion of bovine thyroglobulin revealed that although peptides in the range of 14 to 17 kDa were rapidly generated, these remained stable and were able to activate patient basophils after prolonged gastric digestion.[32] Survival of α-Gal–carrying peptides of sufficient length is a prerequisite for triggering symptoms after passage into the bloodstream.

The main limitation of this study is the lack of food challenges with isolated glycolipids and glycoproteins. For ethical reasons, this is difficult in humans, but it could be done in an α-1,3-galactosyltransferase–knockout mouse model to investigate the parameters responsible for the delayed appearance of symptoms. However, taken together, the findings by us and others provide strong evidence that glycoproteins play a major role in reaction to PK: (1) clinical evidence of PK's being a potent trigger of red meat allergy with symptoms that often are more severe and occur more rapidly,[4,26] (2) high content of α-Gal in PK compared to beef muscle,[24] (3) high content of α-Gal–carrying glycoproteins paired with a very low content of α-Gal–carrying glycolipids, and (4) high stability of α-Gal–carrying glycoproteins during simulated gastric digestion.

In conclusion, we showed that IgE of patients with α-Gal syndrome binds to α-Gal irrespective of the carrier molecule, glycolipid or glycoprotein. Glycolipids and glycoprotein extracts from PK and meat are able to activate basophils, but for PK and beef, glycoproteins seem to be more allergenic *in vitro* than glycolipids, possibly as a result of their higher abundance of α-Gal epitopes. Furthermore, the stability of α-Gal–carrying glycoproteins in PK during gastric digestion suggests that glycoproteins are playing a role in the generation of delayed allergic symptoms. Our findings support the hypothesis that delayed symptoms are related to the late absorption of the allergen, irrespective of the nature of the α-Gal–carrying molecule, glycolipid or glycoprotein. The relative abundance of α-Gal epitopes and the stability of glycolipids and glycoproteins in the respective foods will most likely determine the relevant trigger molecules, depending on the food matrix ingested. Immune responses to protein allergens are well characterized. In contrast, little is known on the immunogenicity and allergenicity of carbohydrates.[33] The carbohydrate α-Gal investigated in this study can be used as a model to further explore the mechanisms involved in the break of tolerance and sensitization to carbohydrate allergens bound to lipids and/or proteins.

We thank Simon Riel, Eberhard Karls University Tuebingen, for excellent technical support and Michel Vaillant, Luxembourg Institute of Health, for assistance with statistics. We thank all patients for participating in the study. We also thank Servier Medical Art for illustrations used in the graphical abstract and ExPASy–Glycodigest for glycan structures drawn using the 'glycodigest' web interface.

Clinical implications: The relative abundance of α-Gal epitopes and the stability of glycolipids and glycoproteins in the ingested food will most likely determine the relevant trigger molecules in α-Gal syndrome.

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METHODS

Patients and sera

The serum of a house dust mite–allergic patient was used as negative control (sIgE to α-Gal < 0.1 kU_A/L, sIgE *Dermatophagoides pteronyssinus* 8.9 kU_A/L, total IgE 72.2 kU_A/L) in ELISA. The α-Gal–positive patient serum pools were constituted from 15 and 21 patients with sIgE to α-Gal (sIgE α-Gal 74.8 and 28.1 kU_A/L, respectively). The first pool relates to patients with α-Gal syndrome recruited outside this study; the second was prepared using sera of 21 patients recruited in Luxembourg. A serum pool of 4 volunteers (without α-Gal sIgE) was used as a negative control in thin-layer chromatography. Six allergic patients (sIgE α-Gal < 0.1 kU_A/L) and 2 healthy volunteers were included as negative controls for BAT.

Extraction of glycolipids and lipids

Glycolipids were extracted from rabbit erythrocytes as described by Galili et al[E1] with some modifications. Briefly, 100 mL of rabbit erythrocytes (Innovative Research) was lysed in water and extracted overnight in chloroform and methanol (3:4, vol/vol); water was then added to 0.2× the final volume to create a Folch partition, which results in the separation of organic and aqueous phases. The aqueous phase was collected and concentrated in a rotary evaporator (Büchi Labortechnik, Flawil, Switzerland), followed by lyophilization in a SpeedVac device (Thermo Electron, Thermo Fisher Scientific). Lyophilized glycolipids were weighed and stored at −20°C. For analysis, they were reconstituted in buffer or water (wt/vol) as required.

Total lipids were extracted from PK, beef, and chicken meat as described by Román-Carrasco et al.[E2] Tissues (3-4 g) were homogenized in water and extracted thrice in chloroform and methanol, and the supernatant was collected. Supernatants were combined, filtered and concentrated, and lyophilized as mentioned above. All dried extracts were stored at −20°C.

Thin-layer chromatography

Extracted rabbit glycolipids and meat lipids were analyzed on 5 × 7.5 cm aluminium-backed silica gel plates (HPTLC Silicagel 60 F_{254}, Merck Millipore, Darmstadt, Germany). Rabbit glycolipids were reconstituted in chloroform:methanol:water (350:480:175, vol/vol/vol), and 20 to 30 μg was spotted onto TLC plates and allowed to air dry. Plates were developed in chloroform/methanol/water (50:40:10, vol/vol/vol) for 60 minutes and air dried. Meat lipid extracts were reconstituted in water, and 30 to 40 μg was spotted onto TLC plates. Air-dried plates were first developed in a chloroform:acetone solution (1:1, vol/vol) for 1 hour, followed by a second development in chloroform:methanol:water (58:34:7, vol/vol/vol) for 45 minutes in a TLC chamber. Three glycolipids were used as reference: Galα-1,3-Galβ1-HDPE, Galβ-1,4-GlcNAcβ1-HDPE, and Galα-1,3-Galβ-1,4-GlcNAc1-HDPE (Dextra Laboratories, Reading, United Kingdom). They were reconstituted in chloroform:methanol:water (350:480:175, vol/vol/vol) and mixed 1:1:1 (vol/vol/vol); then 15 μg of the mixture was spotted onto TLC plates and analyzed.

Carbohydrates were visualized with orcinol (Sigma-Aldrich, St Louis, Mo) and lipids with primulin (Sigma-Aldrich) according to established staining procedures.

TLC immunostaining

For immunostaining, a modified protocol by Magnani et al[E3] was used. Plates developed as previously described were air dried and coated with 0.5% poly isobutyl methacrylate (Sigma-Aldrich) in hexane. The plates were allowed to air dry and blocked with 1% HSA (Sigma-Aldrich) in Tris-buffered saline with 0.05% Tween 20 (hereafter HSA/TBST), pH 7.5. The α-Gal epitopes were detected using either a murine monoclonal anti–α-Gal IgM (clone M86; Enzo Life Sciences) or an α-Gal–allergic patient serum pool (Table E1) diluted 1:10 either in 1% (anti–α-Gal IgM) or 0.5% (patient serum pool) HSA/TBST. Plates were incubated overnight at 8°C with gentle shaking. The next day, plates were washed and bound antibodies detected with either anti-mouse IgM-AP (Southern Biotech, Birmingham, Ala) or anti-human IgE-biotin (Southern Biotech). Plates incubated with anti-human IgE-biotin were further incubated with streptavidin-AP (BD Biosciences, Allschwil, Switzerland). Anti–α-Gal antibody binding was visualized by color development with nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl-phosphate (NBT/BCIP, Promega, Leiden, The Netherlands).

Glycan analysis

Glycolipids extracted from rabbit erythrocytes and PK were analyzed by Asparia Glycomics according to their established protocol.[E4] In short, glycans were enzymatically (EGCase I, New England Biolabs, Ipswich, Mass) removed and labeled with the fluorescent tag 2-aminobenzamide. Labeled glycans were separated by ultra performance liquid chromatography (Acquity UPLC system, Waters, Milford, Mass) using a hydrophilic interaction liquid chromatography column (Acquity UPLC Glycan BEH Amide, Waters), and relative quantification was performed with a coupled fluorescent detector. A second detection of released glycans by MALDI-TOF MS (UltrafleXtreme, Bruker Daltonics, Bremen, Germany) allowed glycan identification based on previously reported structures

For MALDI-TOF MS/MS analysis of intact glycolipids and determination of their different glycan connectivity, intact glycolipid samples were diluted in restriction enzyme buffer (New England Biolabs) and analyzed by MALDI-TOF MS. Fragmentation spectra (MS/MS) were acquired for the most representative ions observed in MALDI-TOF MS by in-source fragmentation using the LIFT application implemented by the Bruker UltrafleXtreme MALDI-TOF equipment. Annotation of the different carbohydrate fragments was performed by Flex Analysis software (Bruker). Linkage confirmation is possible as a result of the individual signatures of each fragment, as observed in the mass spectra.

Protein extraction

For the digestion assay, PK tissue (1 g) was cut into small pieces approximately 2 × 2 mm in size, then placed into a 2 mL plastic tube. Cooked PK was incubated for 20 minutes at 95°C in a water bath. Two steel beads, 5 mm in diameter, were added to each tube of cooked and raw PK, and the tube filled up to 2 mL with 1× simulated salivary fluid.[E5] Tubes were placed in an MM400 tissue lyser (Retsch, Haan, Germany) for 2 × 2 minutes at 25 Hz. These extracts are referred hereafter as PK tissue extract.

Protein extracts of PK and beef meat used for sodium dodecyl sulfate–polyacrylamide gel electrophoresis, BAT, and ELISA were prepared as described above. A total of 100 mg tissue was lysed in 2 mL Tris (50 mmol/L, pH 7) supplemented with protease inhibitor cocktail (11836170001, Roche, Basel, Switzerland). Lysed tissues were centrifuged at 15,000*g* at 4°C for 10 minutes; then the supernatant was collected and stored at −20°C. Protein concentrations were determined by Bradford protein assay (500-0006, Bio-Rad, Temse, Belgium).

Dynamic light scattering

Glycolipids form micelles containing a hydrophobic core and a hydrophilic corona when dissolved in a polar solvent such as water or phosphate-buffered saline (PBS). The size and polydispersity of the formed micelles were determined by dynamic light scattering (Zetasizer Nano ZS, Malvern Panalytical, Malvern, United Kingdom). Glycolipids and lipid extracts were dissolved in PBS at a concentration of 5 μg/mL and analyzed.

Detection of sIgE by ELISA and relative α-Gal quantification by inhibition ELISA

Nunc Maxisorp (Thermo Fisher Scientific) plates were coated overnight at 4°C with rabbit glycolipids extracted from erythrocytes or α-Gal HSA (Dextra Laboratories) at a concentration of 5 μg/mL in PBS. The next day, the wells were blocked with 1% HSA/TBST, and after washing, 100 μL of patient sera (diluted 1:5 to 1:200 in 0.5% HSA/TBST) was added and incubated overnight at 4°C. Bound IgE was detected using biotin-conjugated anti-human IgE (Southern Biotech), followed by incubation with streptavidin-AP (BD Biosciences), and signal was developed with p-nitrophenyl phosphate (Sigmafast, Sigma-Aldrich) as substrate. Optical density (OD) measured at 405 nm. OD values obtained by a negative control serum were subtracted from the patient OD values for data analysis.

The relative quantification of α-Gal epitopes was established by inhibition ELISA. Briefly, MaxiSorp plates (Thermo Fisher Scientific) were coated overnight with 2 μg/mL of α-Gal HSA in PBS at 4°C and blocked with 1% HSA/TBST. PK and beef (protein and lipid) extracts, rabbit glycolipids, and α-Gal HSA were diluted in PBS to reach a final concentration of 200/20/2/0.2/0.02 μg/mL and were incubated 1:1 (vol/vol) with an α-Gal–allergic patient serum pool (diluted 1:20 in PBS) for 2 hours at room temperature. Inhibited samples were added to the microtiter plate wells and incubated overnight at 4°C. The next day, bound IgE was detected as described above. A similar procedure was followed to analyze the inhibition of anti–α-Gal mouse monoclonal antibody M86 (dilution 1:50 in PBS) by PK and beef lipids.

Recombinant AP-N and polyclonal rabbit antiserum

Ectodomain of AP-N (UniprotKB P15145, AMPN_PIG; aa 33 to aa 963) was inserted into a pBudCE4.1 (V532-20, Invitrogen, Life Technologies, Carlsbad, Calif) vector, then fused with signal peptide for expression and a polyhistidine (6xHis) tag for purification. The vector was transfected into HEK 293 cells and positive clones screened using Zeocin as a selection marker, and a stable cell line was established. Thereafter, AP-N was expressed and secreted into the cell culture medium, then purified by immobilized metal-ion affinity chromatography and ion exchange chromatography (Mono Q, GE Healthcare, Waukesha, Wis).

Rabbit antiserum was produced by Eurogentec (Liège Science Park, Seraing, Belgium) by immunizing 2 rabbits with the recombinant aminopeptidase N as per their established protocol and IgG purified.

Simulated gastric and intestinal digestion

Gastric digest. Simulated gastric fluid (SGF) was prepared as a 1.25× solution at pH 3.[E5] Pepsin (P6887, Sigma-Aldrich) at 3200-4500 U/mg resuspended in SGF 1.25×, pH 6.4, to obtain a stock solution of 25000 U/mL (5.5-7.8 mg pepsin/mL SGF 1.25×). At pH 6.4, pepsin is inactive, thereby preventing autodigestion. The stock solution was freshly prepared before each assay.

Protein extract, SGF 1.25× (pH 3), pepsin stock solution, and ultrapure water were preheated for a few minutes at 37°C in a thermo shaker (dry bath) and mixed as followed to obtain 0.5 mL of SGF 1× at pH 3 with pepsin: 320 μL SGF 1.25× (pH 3), 96.5 μL ultrapure water, 80 μL pepsin stock solution, and 6.5 μL HCl 2 mol/L. Next, 0.5 mL protein extract at 5 mg/mL was added (1:1, vol/vol) and PK tissue extract (raw or cooked) added 1:1 (vol/vol). The final simulated gastric digestion mixture had pepsin activity of 2000 U/mL. The simulated gastric digest was incubated at 37°C in a thermo shaker and an aliquot withdrawn after the indicated time intervals. The reaction was stopped by adding 1.5 μL NaOH 1 mol/L to each 50 μL aliquot to inactivate pepsin.

Intestinal digest. Simulated intestinal fluid (SIF) was prepared as a 1.25× solution at pH 7.[E5] Porcine bile extract (B8631, Sigma-Aldrich) dissolved in ultrapure water at 100 mg/mL and total bile acids titrated with total bile acid assay kit (DZ092A-K, Diazyme, Dresden, Germany). The concentration of bile acids in the stock solution ranged from 130 to 160 mmol/L. Pancreatin from porcine pancreas 8× USP specifications had a protease activity of 200 U/mg (P7545, Sigma-Aldrich). Stock solution of pancreatin was prepared fresh at 800 U/mL in SIF 1.25× and kept on ice to prevent proteolytic autodigestion. The units of pancreatin refer to protease activity of trypsin only.

SIF 1.25×, bile extract, $CaCl^2$ 0.3 mol/L, and ultrapure water were preheated a few minutes at 37°C in a thermo shaker and mixed as followed to obtain 0.4 mL of SIF 1× at pH 7: 220 μL SIF 1.25×, 50 μL bile extract, 26.2 μL ultrapure water, 0.8 μL CaCl2 0.3 mol/L, 3 μL NaOH 1 mol/L, and 100 μL pancreatin. Then 0.4 mL gastric chyme was added (1:1, vol/vol). The final simulated intestinal digestion mixture had a trypsin activity of 100 U/mL and a final concentration of 10 mmol/L bile acids. The simulated intestinal digest was incubated at 37°C in a thermo shaker and an aliquot removed at the indicated time intervals. The reaction was stopped by heating the aliquot immediately at 95°C for 5 minutes to inactivate the pancreatic protease activity, then freezing at −20°C.

Basophil activation test

The Flow CAST (Bühlmann Laboratories) assay was used for quantitative measurement of *in vitro* basophil activation. Venous blood was collected from 19 patients using 10 mL EDTA blood collection tubes and the assay performed within 24 hours, as described elsewhere.[E6] Briefly, increasing concentrations of allergen extract (in 50 μL) were added to the polystyrene tubes and diluted with 100 μL stimulation buffer (containing heparin, Ca^{2+}, and IL-3 [2 ng/mL]). Patient whole blood (50 μL) was added, followed by 20 μL staining reagent (anti-CD63–fluorescein isothiocyanate and anti-CCR3–phycoerythrin mAbs). Incubation, lysis, and wash steps were performed according to the manufacturer's protocol. Relative cell count of CD63-positive basophils was determined by flow cytometry (FACSCanto flow cytometer, Becton Dickinson, San José, Calif).

Statistical analysis

ELISA data were analyzed by GraphPad Prism v9 (GraphPad Software, La Jolla, Calif) using the Wilcoxon matched-pair signed-rank test or nonlinear regression with a 3-parameter analysis where applicable. BAT data from different allergen extracts were compared using relative EC_{50} value and area under the curve. Briefly, relative EC_{50} values were estimated from 4-parameter log-logistic models fit to each allergen data point. Goodness of fit was compared between different models to assess the quality of the models. Pairwise comparisons of EC_{50} values were performed by the ratio test.[E7] Bonferroni correction was applied to account for multiple testing. One-way ANOVA Welch test of equivalence of areas under the curve was performed, followed by multiple *post hoc* Welch 2-sample *t* tests. Bonferroni correction was applied at the stage of *post hoc* testing. All tests were 2 tailed and were performed at the 5% significance level. Basophil activation data were analyzed by R v4.0.3 (www.r-project.org) and GraphPad Prism v9.

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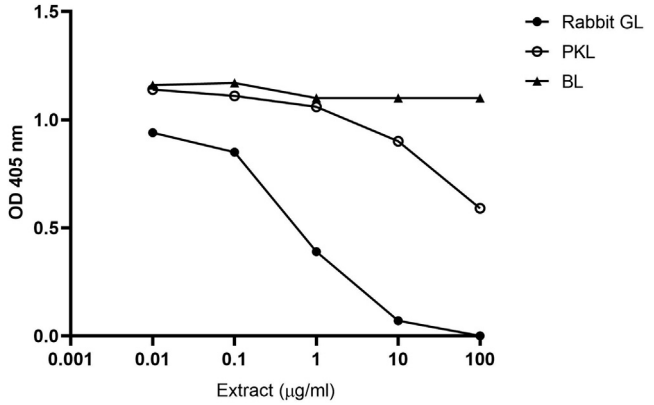

FIG E1. Detection of α-Gal–carrying glycans in lipid extracts with anti–α-Gal mouse monoclonal antibody M86. For inhibition ELISA, α-Gal-HSA (2 μg/mL) precoated onto a microtiter plate was detected via M86, which was previously inhibited with extracts (*BL,* beef lipids; *GL,* rabbit glycolipids; *PKL,* PK lipids) at varying concentrations (0.01-100 μg/mL).

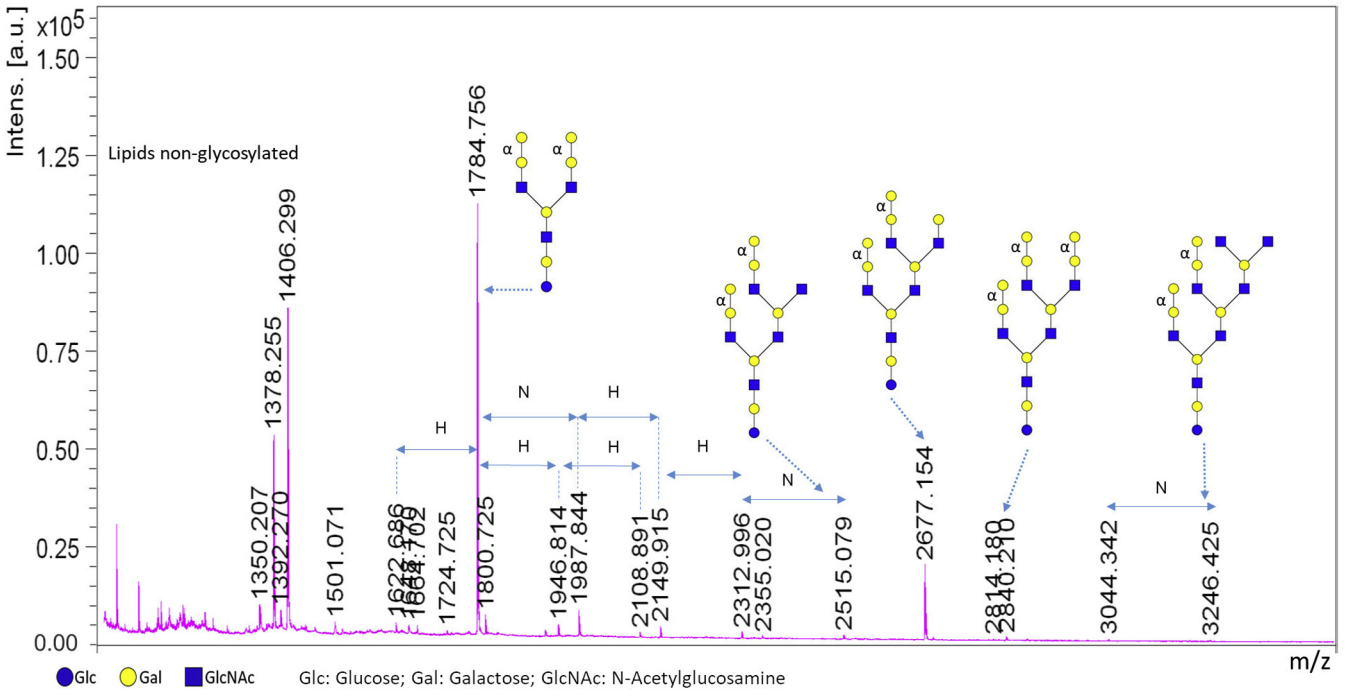

FIG E2. Numerous glycans with terminal α-Gal epitope detected in rabbit erythrocyte extract (rabbit glycolipids) via MALDI-TOF MS–based structure determination of glycans and glycomic analysis.

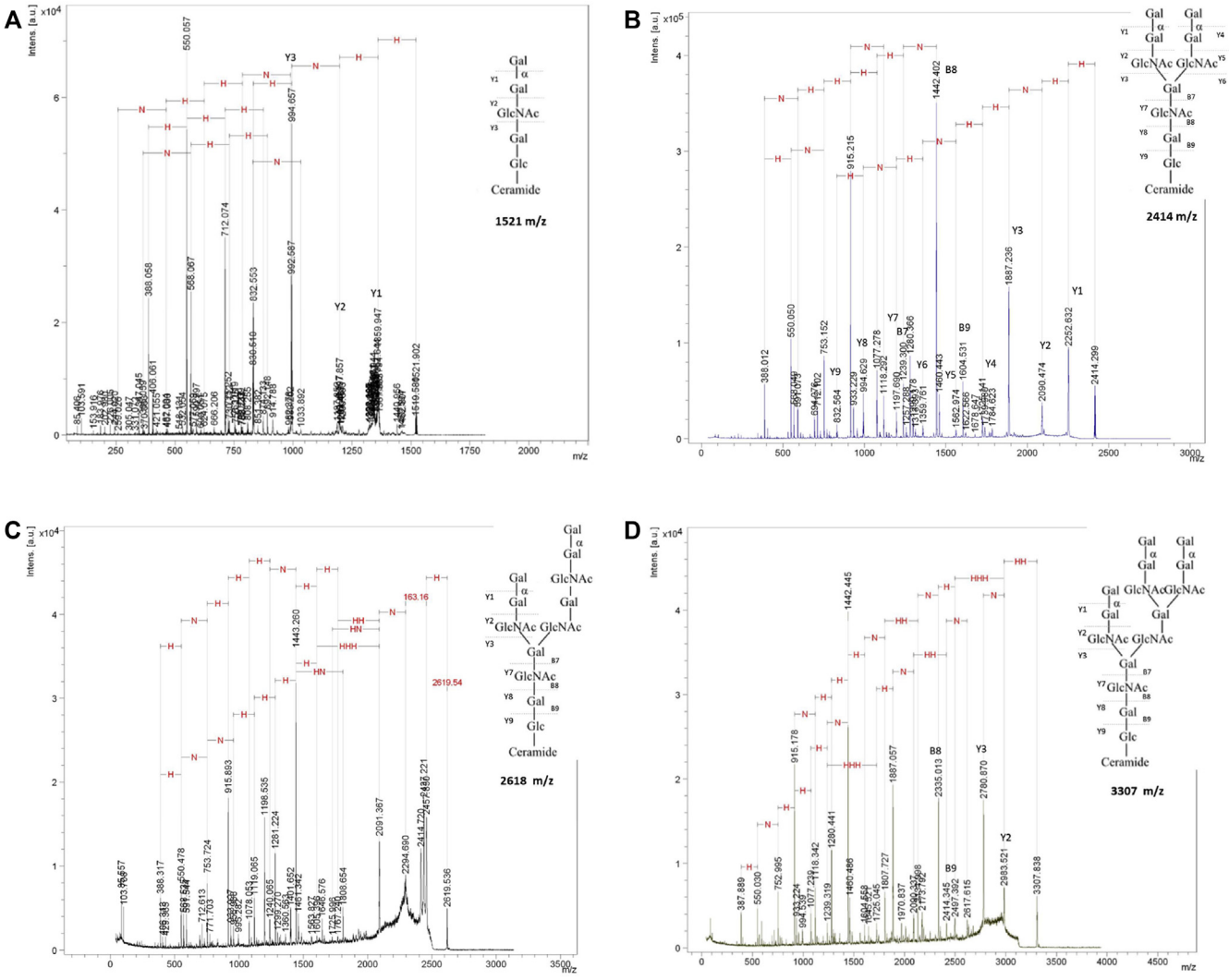

FIG E3. MALDI-TOF MS/MS analysis of rabbit glycolipids. Spectra show α-1,3 linkage determination in glycans from 4 α-Gal–carrying rabbit glycolipids.

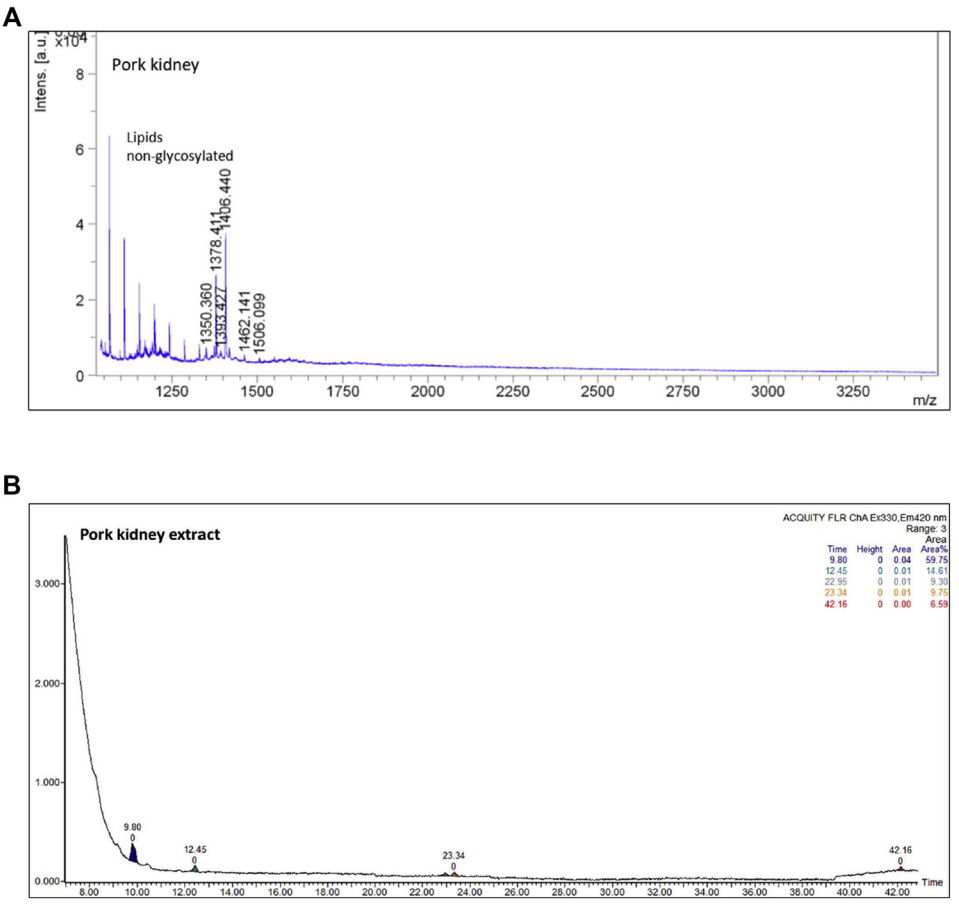

FIG E4. Glycomic analysis of PK lipid extract failed to detect α-Gal–carrying glycans as a result of their low abundance. (**A**) MALDI-TOF MS–based identification of glycans; *x-axis* indicates m/z; *y-axis,* intensity (AU, arbitrary units) $\times 10^4$. (**B**) Relative quantification of glycolipids via ultra performance liquid chromatography coupled to fluorescence detector; *x-axis* indicates time (minutes); *y-axis,* EU.

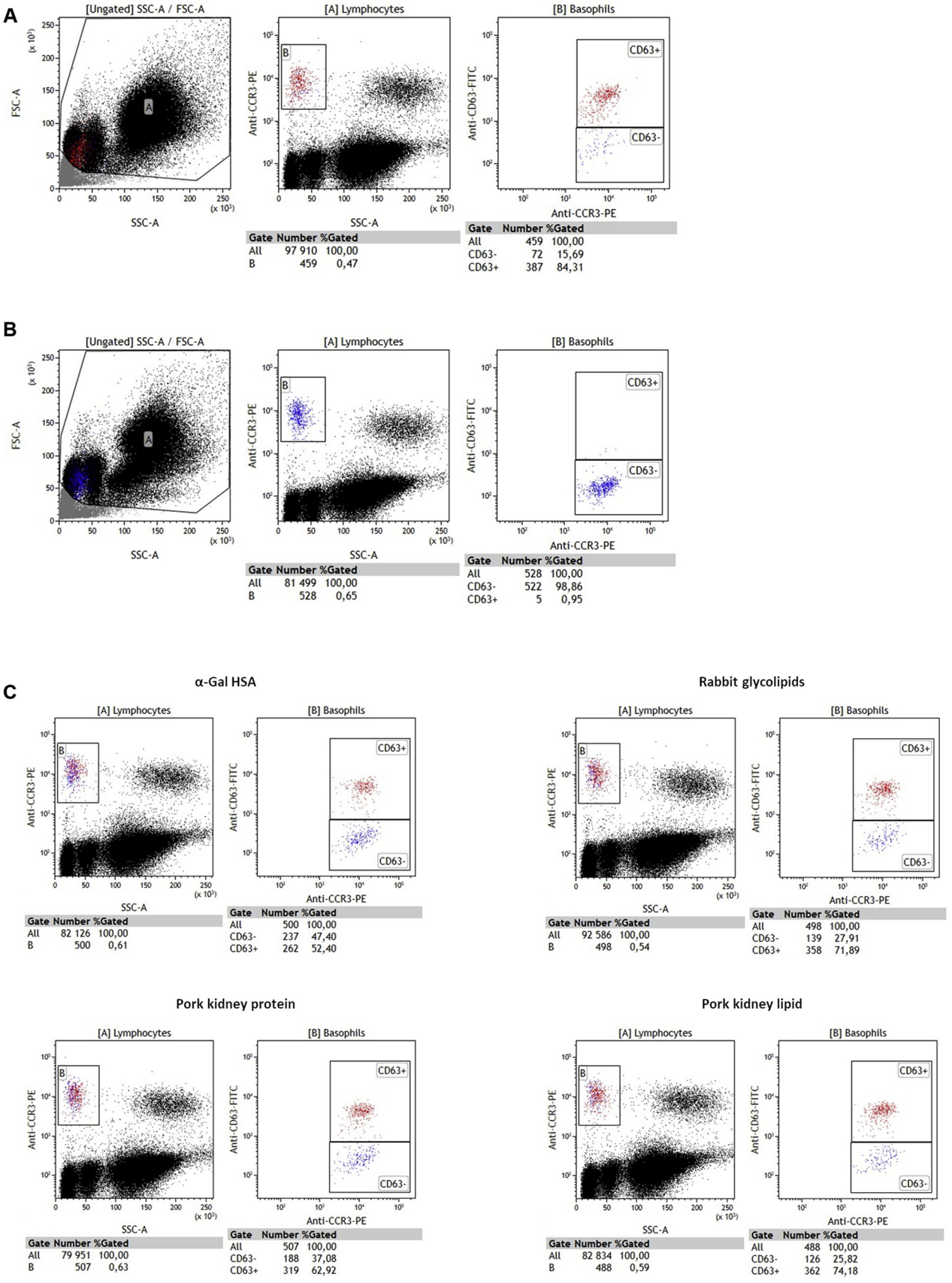

FIG E5. Basophil gating strategy and activation plots. (**A**) Positive control (anti-FCεRI). (**B**) Negative control (unstimulated). (**C**) Scattered dot plots showing upregulation of CD63 obtained after stimulation with α-Gal HSA (10 μg/mL; *top left*), rabbit glycolipids (10 μg/mL; *top right*), PK protein (100 μg/mL; *bottom left*), and PK lipids (100 μg/mL; *bottom right*); patient sIgE (α-Gal) = 15 kU_A/L and total IgE = 1006 kU/L.

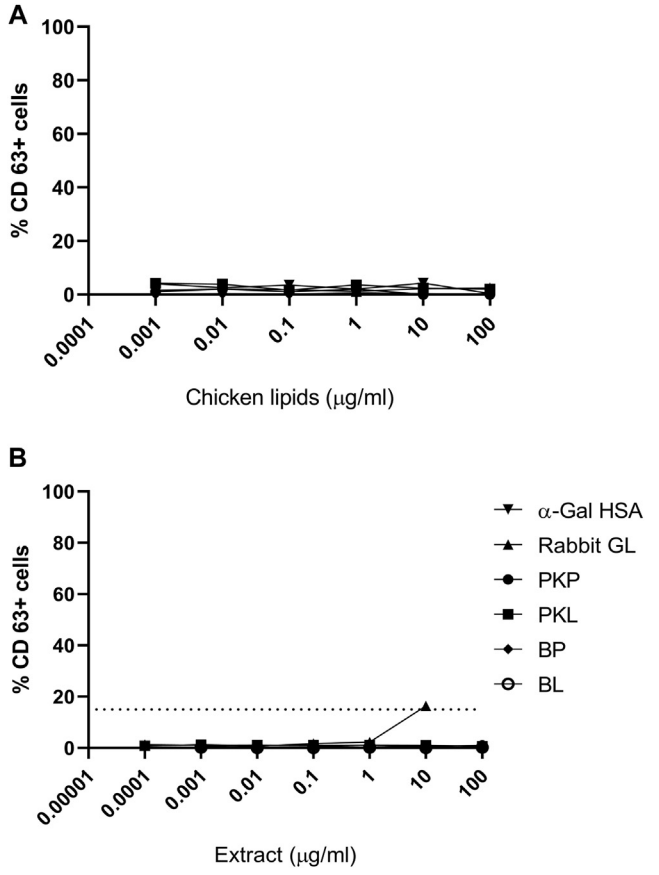

FIG E6. (**A**) Basophils from clinically reactive α-Gal–allergic patients (n = 5; sIgE 0.91-50.8 kU_A/L) are not activated on incubation with chicken lipids at varying concentrations. Percentage of CD63-positive cells (*y-axis*) vs chicken lipid extract (*x-axis*). (**B**) Basophils of allergic or healthy controls (α-Gal sIgE < 0.1 kU_A/L) are not activated on incubation with α-Gal HSA, rabbit glycolipids (*GL*), PK (*PKL,* PK lipid; *PKP,* PK protein) (n = 5), or beef (*BL,* beef lipid; *BP,* beef protein) (n = 3) at varying concentrations. Data are medians of all patients (percentage CD63-positive cells) at each concentration per extract plotted (*y-axis*) against increasing dose (*x-axis*). *Dotted line* indicates positivity cutoff for food allergens (15%).

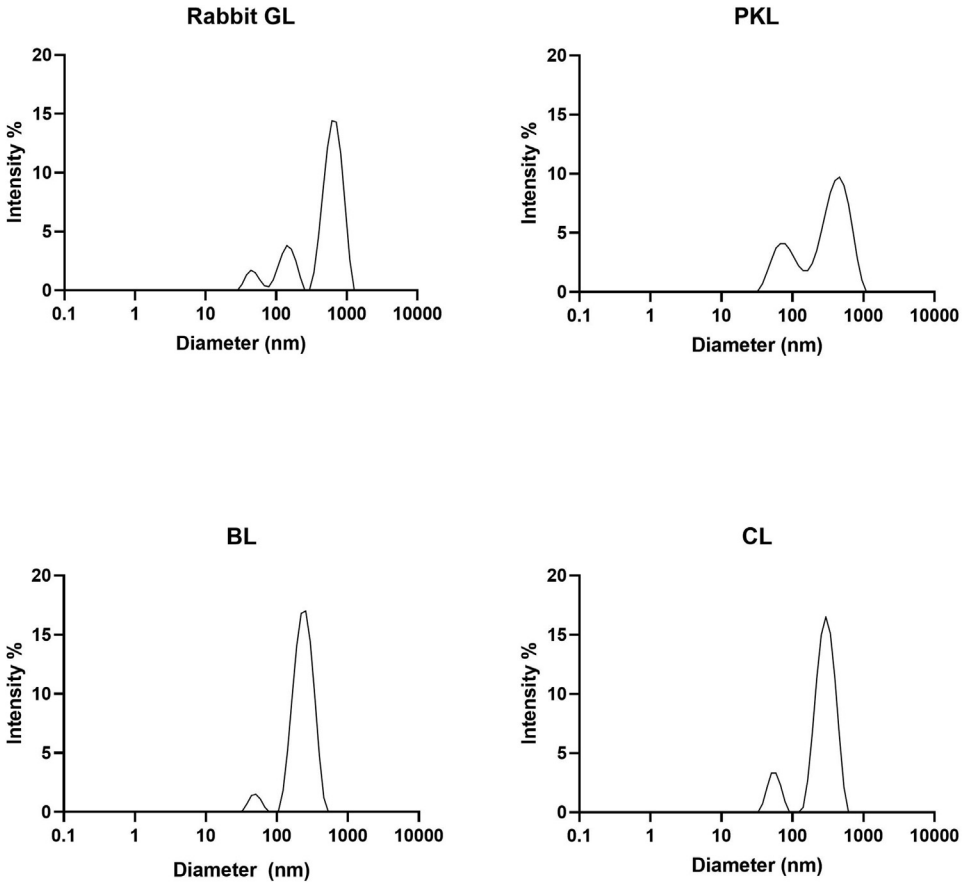

FIG E7. Glycolipids form micelles when dissolved in a polar solvent. Micelles formed by rabbit glycolipids (*GL*) and PK lipid (*PKL*), beef lipid (*BL*), and chicken lipid (*CL*) extracts as observed by dynamic light scattering. *X-axis* indicates diameter of micelles (nm); *y-axis,* percentage intensity.

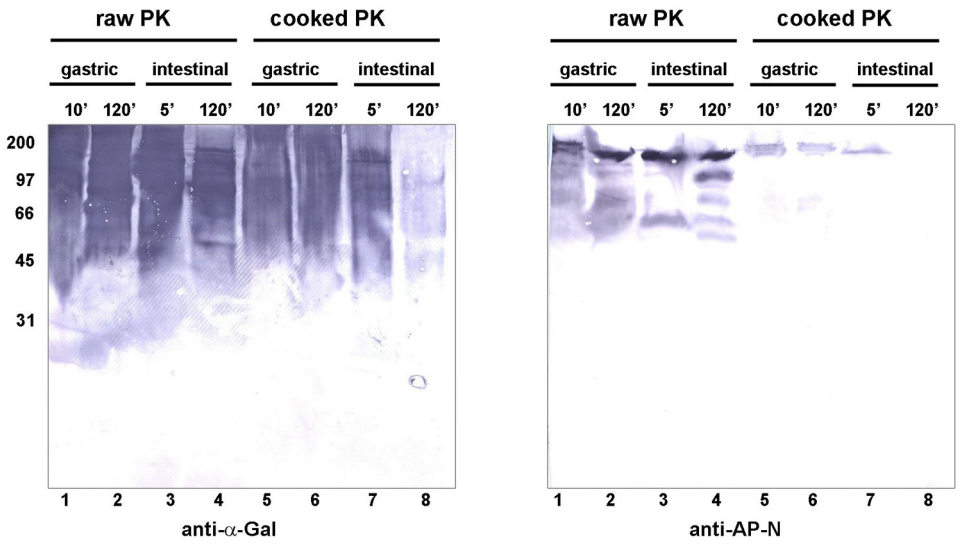

FIG E8. α-Gal–carrying glycoproteins in PK are stable under gastric digestion conditions. Complete PK extract (soluble and insoluble proteins) immunoblot of simulated gastric and intestinal digest from raw and cooked PK; detection with anti–α-Gal and anti–AP-N antibodies. Time point of sample analysis is indicated above each lane.

TABLE E1. Demographic and clinical data from patients with sIgE to α-Gal

Patient no.	Age (years)	Sex	Trigger for anaphylactic reaction	Response delay (hours)	Symptoms related to α-Gal	Anaphylaxis severity grade*	Positive skin test result (prick/ic)	α-Gal sIgE (kUA/L)	IgE titer (kU/L)
1	52	F	Spare ribs (pork), lamb	5	U, dyspnea	II	P, B, PK, BK, G	14.0	443
2	51	F	Beef	6	U	I	P, B, PK, BK, G	5.1	125
3	37	M	Beef kidney	0.5	U	I	P, B, PK, BK, G	0.81	122
4	81	F	PK	3	U, dyspnea, F	III	P, B, PK, BK, G	0.49	60.9
5	53	F	Milk, cheese	0.5	U, dyspnea, Ap	II	P, B, PK, BK, G	31.5	821
6	79	M	PK	2	U, circulatory shock	II	P, B, PK, BK, G	37.4	678
7	60	F	PK	1-2	U, dyspnea	II	P, B, PK, BK, G	20.2	96.5
8	64	F	Pork, beef	3	U	I	P, B, PK, BK, G	3.0	115
9	18	M	Sausages	3	U	I	P, B, PK, BK, G	34.4	305
10	63	M	PK	6	U	I	P, B, PK, BK, G	7.1	228
11	59	M	PK	5-6	U, circulatory shock, F	III	P, B, PK, BK, G	20.1	86.2
12	56	M	PK, pork, beef, deer, gelatin	0.5, 3-6	U, dyspnea, circulatory shock, F	III	P, B, PK, BK, G	50.8	3142
13	73	F	PK, pork, beef	NA	U, dyspnea, D	II	PK, G	2.2	8.7
14	24	F	Pork, beef	2-3	U	I	P, B	9.4	36.5
15	67	M	No clinical symptoms	NA	NA	NA	NA	4.7	175
16	63	M	No clinical symptoms	NA	NA	NA	NA	0.91	155
17	84	M	No clinical symptoms	NA	NA	NA	NA	9.0	1857
18	67	F	No clinical symptoms	NA	NA	NA	PK, BK	4.7	314
19	70	M	Pork, beef	20-60 min	U, F	I	PK, BK, B	46	800
20	21	M	Beef	6-8	U, Ap	I	PK , B	3.8	ND
21	43	M	Pork, beef, mutton	3-4	U, cough, wheezing, hypotension	III	ND	138	562
22	13	M	Pork meat	2-3	Ap, U, V, heart palpitations	III	P, PK	69	765
23	19	M	Sport	1-4	U, nausea and Ap (1 episode)	I	B, PK	5.52	80
24	29	F	Ground meat, ham, sausage	1-6	U, palpebral AE	I	B, PK	1.55	72
25	31	F	Cheeseburger or cheese	3-4	U, lingual AE	I	B, P, PK	2	18
26	46	M	Ground meat, sausage, cheese	3-4	U	I	B, PK	18	32
27	48	M	Sausages	2-6	U, V, D, labial or laryngeal AE	II	B, P, PK	12	126
28	20	M	Ice cream, cheese, gelatin	ND	Ap, D	II	P, B, PK	1.2	168
29	20	M	Bolognese or cheese	1-4	Ap, V, F	I	PK	13	143
30	52	F	Dairy products	ND	Acute U	I	PK, milk	0.96	42
31	48	F	ND	Overnight	Recurrent U, palpebral AE	I	B, PK	21	190
32	36	F	No clinical symptoms	NA	NA	NA	B, P , PK, milk	1.25	122
33	26	F	No clinical symptoms	NA	NA	NA	B	2.09	221
34	62	M	Intravenous infusion beef gelatin	Immediate	Perioperative anaphylaxis	IV	PK	18	4993
35	27	M	Beef meat and other red meats, exacerbation with alcohol	4-6	U, Dy, Wh	II	B, P, PK	66	373
36	32	M	Beef and pork meat	3-6	Acute U, Dy, C, laryngeal dysphagia	II	B, P, PK	27	401
37	56	M	Red meat with ace inhibitor	Immediate	Angioedema	ND	PK, B	>100	>1000
38	35	M	Fat meal	30 min	Swelling of throat	ND	PK	0.63	205
39	45	M	Lamb, ham	4-6	U, pruritus, mild Brsp	ND	PK, P, B	63	639

AE, Angioedema; *Ap,* abdominal pain; *B,* beef meat; *BK,* beef kidney; *Bm,* bowel movement; *Bp,* breathing problems; *Brsp,* bronchospasm; *D,* diarrhea; *F,* faintness; *G,* gelatin (intracutaneous); *Hy,* hypotonia; *NA,* not applicable; *ND,* not defined; *P,* pork meat; *S,* syncope; *U,* urticaria; *V,* vomiting; *W,* weakness.

*Severity grade of anaphylaxis according to: Ring J, Behrendt H. Anaphylaxis and anaphylactoid reactions. Classification and pathophysiology. Clin Rev Allergy Immunol 1999;17:387-99.

CHAPTER IV

Chapter IV. Role of spatial distribution and epitope number on IgE-effector function in allergic response against α-Gal

INTRODUCTION

Most allergies known to date are caused by protein allergens. Symptoms occur when peptide epitopes bind to IgE on the surface of effector cells (such as basophils, mast cells etc.) resulting in crosslinking and cellular activation [1]. Peptide epitopes recognized by specific IgE antibodies are a sequence of amino acids (usually 6-15 amino acids) present either in a conformational or linear configuration [2]. Carbohydrate-mediated allergies present a new challenge to allergen characterization due to the difference in arrangement of glycans in a carbohydrate structure compared to peptide epitopes in a protein molecule. In protein allergens, amino acids are added sequentially during translation and the protein molecule is folded into a 3D configuration in the endoplasmic reticulum along with various other co-translational/post-translational modifications [3]. Carbohydrates on the other hand are a product of the activity of various glycosyltransferases which add a glycan structure at a specific linkage, and glycosylation depends upon availability of donor and acceptor substrate as well as competition for sugars with other biosynthesis processes [4, 5]. Therefore, resulting in a huge variation in glycan synthesis that makes their study quite complex. Another important characteristic is the presence of α/β linkage at different positions, altering glycan structure and therefore making their study even more complex [6, 7].

There are few structural studies of carbohydrate epitopes in the context of allergic responses due to the almost negligible role of carbohydrates in allergy, except as cross-reactive carbohydrate determinants (CCDs). These CCDs belong to helminths, insects, and plants, and most of them reported to date are from plants [6]. Although cross-reactive carbohydrates are widely known in the field of allergy they are not considered to be of much clinical interest due to their low or no clinical relevance. Owing to their wide occurrence and presence in many allergen sources, they often contribute to false positive results in *in vitro* IgE-diagnosis when using allergen extracts or native molecules, but they do not induce allergic response in patients by themselves [8]. An important role of carbohydrates in allergy emerged with the description of red meat allergy and its association with IgE directed to the α-Gal epitope. The involvement of a carbohydrate allergen calls for a better understanding of molecular interaction to devise optimized diagnostic tools and preventive measures. The α-Gal epitope is present on both glycoproteins and glycolipids and an important aspect is to understand the role of carrier molecules in allergenic response [9-16].

The physicochemical properties of allergen molecules as well as the quantity and polyclonality of specific IgE in serum are known to determine the allergenic potency of an allergen molecule [17, 18], in addition recent studies have found that the number of epitopes and their proximity on the allergen molecule are a major factor contributing towards determining allergenicity [19]. Structural analysis of several prominent allergens have revealed the presence of multiple IgE-binding sites on allergens, for example twenty-one IgE-binding epitopes have been identified in Ara h 1, ten epitopes in Ara h 2 and four in Ara h 3 [20-22]. Other major allergens with mapped multiple linear epitopes are shrimp, milk, soy, and cashew [23-27]. The epitope proximity and number determines the shape of immune complexes formed by the binding of the allergen molecule to specific IgE. Gieras et al. have shown a clear impact of the number and proximity of IgE-binding sites present on an allergen molecule on effective cross-linking of IgE antibodies and subsequent effector cell degranulation by using model allergens. They built models by grafting multiple sequential epitopes of an allergenic peptide of Phl p 1 (timothy grass pollen), a common respiratory allergen, onto a model protein (horse heart myoglobin) at varying distances [19]. Although well understood in case of protein molecules, the structural requirements on IgE cross-linking by carbohydrate epitopes remain to be characterized.

Aminopeptidase N (AP-N) also known as CD13 was previously recognized in our group as a major α-Gal-carrying IgE-reactive allergen from pork kidney (Hilger et al. 2016 [28], see figure 1), a potent trigger of allergic reactions in α-Gal-allergic patients. Patient IgE specifically recognized α-Gal present on the AP-N molecule, and AP-N was found to be highly allergenic in basophil activation (see figure below, part A) and skin prick test. It is a widely expressed and heavily glycosylated surface-anchored metallopeptidase found in tissues such as intestinal epithelia and brush-border membranes of kidney epithelial cells. It plays a pivotal role in functions such as peptide metabolism, viral receptor, and signal transduction etc. [29].

The aim of this study was to create mutants of porcine AP-N with three glycosylation sites present on each mutant at varying proximity in order to analyse IgE-binding and immune-complex formation. The mutant models will help to understand underlying structural determinants of a carbohydrate allergen leading to IgE cross-linking and effector cell activation. A major requirement for cellular activation by protein allergens is the presence of at least 2 epitopes on an allergen molecule. Therefore, it is highly interesting to understand structural characteristics of α-Gal-glycan that make it allergenic.

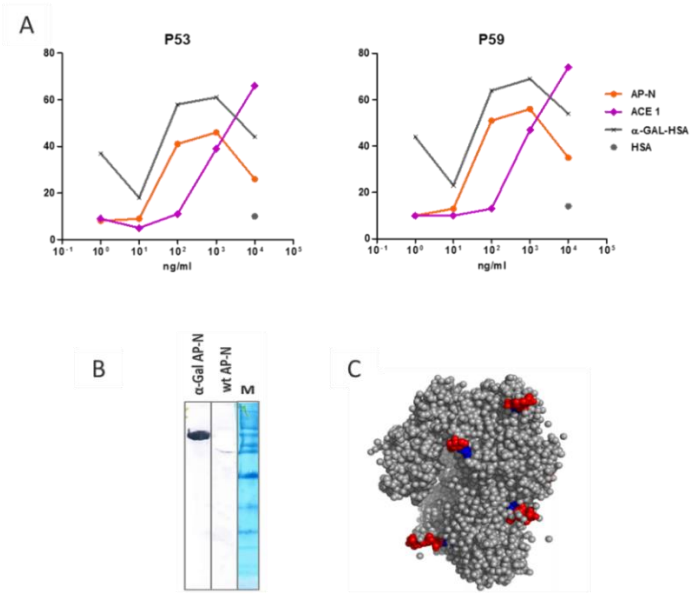

Figure 1. Porcine aminopeptidase N (AP-N): A. Basophil activation assay with AP-N, ACE I (angiotensin-converting enzyme I) in whole blood from two α-Gal-allergic patients, α-Gal-HSA as positive control and HSA as negative control. X-axis – allergen concentration, y-axis – % CD63 positive cells. B. Immunoblot with anti-α-Gal antibody (M86) showing α-Gal-glycosylation on AP-N produced in HEK cells with α-1,3-GT activity (α-Gal AP-N) and absence of α-Gal in AP-N produced in regular HEK cells (wt AP-N). C. Space-fill model of porcine AP-N as visualized in PyMol, red shows glycosylation present in the PDB (id: 4FKK) crystal structure. Figure part A adapted from Hilger et al. [28]

Porcine AP-N used in this study is a transmembrane protein with a short intracellular tail, an anchor, and a small stalk attached to a large ectodomain. It has fourteen potential N-linked glycosylation sites, out of which 13 were glycosylated as visualized in the crystal structure of AP-N [29] (PDB id: 4FKK, see figure 1C). Upon treatment with α-Galactosidase all the α-Gal epitopes were lost, showing absence of α-Gal on any other site except N-linked positions (figure 1B). The mutant molecules were produced in a genetically modified HEK cells (HEK 293-GT) that imparts α-1,3-glycosylation at N-linkage position (N-X-S/T) due to the active gene α-1,3-GT (figure 1B).

MATERIALS AND METHODS

Aminopeptidase N (AP-N) mutants

For selection of sites to mutate, the crystal structure of the porcine aminopeptidase N (PDB: 4FKK) was visualized in PyMol and glycosylation sites were selected based upon residue distance. The AP-N plasmid (APN gene in pBudCE4.1 vector) was kindly provided by Dr. Simon Blank, Helmholtz Center Munich. The plasmid was transformed into TOP-10 *E.coli* cells and a glycerol stock was prepared and stored at -80°C for future use. For mutagenesis, cell culture medium (low salt Luria-Bertani broth) with zeocin (50 µg/ml; Invitrogen), to selectively allow growth of AP-N expressing cells, was inoculated with AP-N culture and incubated overnight at 37°C with shaking (200 rpm), next day plasmids were extracted (Qiagen maxiprep kit) and used in site-directed mutagenesis reaction. Briefly, site-directed mutations were introduced into the APN gene using a commercial kit (QuikChange Multi Site-directed mutagenesis kit, Agilent) and following manufacturers suggested protocol and using 100 ng of AP-N plasmid and 100 ng of each primer. Primers used in the experiment were ordered from Eurofins Genomics. For mutagenesis, following mix was prepared on ice:

Reaction buffer	2.5 µl
Double-distilled H_2O	final vol. 25 µl
QuikSolution	0.5 µl
APN plasmid	100 ng
Primers	100 ng (each primer)
dNTP mix (100 nM)	1 µl
QuikChange multi enzyme mix	1 µl

Incubations were run on a PCR (polymerase chain reaction) thermal cycler (Biorad) using the following amplification program:

95°C	1 min	
95°C	1 min	30x
55°C	1 min	
65°C	16 min	
8°C	hold	

The amplified PCR products were treated with Dpn I (37°C, 1hr) to digest methylated and hemimethylated DNA and were thereafter analysed on agarose gel by electrophoresis. The plasmids were then transformed into XL10-Gold *E.coli* cells and plated on agar plates with zeocin (50 µg/ml), successful transformations were controlled with blue-white screening. After mutation, single colonies

of mutant AP-N clones were picked and cultured. Thereafter plasmids were extracted and amplified as mentioned before followed by sequencing on a cycle sequencer using Big Dye (BigDye™ Terminator v3.1, Applied Biosystems), as per established sequencing protocol (manufacturer's reference guide). Lastly, mutants were analysed on SnapGene viewer and positive clones were selected and used in the subsequent mutation cycles.

Blue-white screening: To control mutagenesis reaction blue-white screening was performed. The manufacturer (Quikchange) provided a plasmid encoding for inactive (mutated) *LacZ* gene to use as a control mutagenesis experiment. The *LacZ* gene product leads to formation of blue colonies in suitable *E.coli* transformants when grown on media supplemented with X-gal (5-Bromo-4-Chloro-3-Indolyl β-D-Galactopyranoside, Invitrogen), a substrate for β-galactosidase, in the presence of IPTG (Isopropyl β-D-1-thiogalactopyranoside, Invitrogen) as inducer. The control plasmid has three mutations in the *LacZ* gene and therefore produces white colonies, and blue colonies are produced when the gene is mutated back to the active *LacZ* using 3 control primer templates and hence was used as a control mutagenesis reaction to monitor efficiency of the mutation reactions.

E.coli transformation

For introduction of plasmid into *E.coli*, cells were thawed on ice for 5 minutes and 50 ng of plasmid was added. The mix was incubated on ice for 30 minutes followed by heat shock at 42°C for 30 seconds and re-incubation on ice for 2 minutes. Thereafter, pre-warmed (37°C) 250 µl of SOC medium (ThermoFischer Scientific) was added and cells were incubated at 37°C for 1 hour at 225 rpm. Cells were then plated (100 µl culture) on low salt LB (Luria-Bertani, Sigma Aldrich) agar plates with zeocin (50 µg/ml) and grown overnight at 37°C.

Protein expression and purification

The mutant AP-N protein was expressed in HEK293-GT cells which were a kind present by Dr. Simon Blank. The cells are genetically modified to exhibit α-1,3-GT (α-1,3-galactosyltransferase) activity in order to impart α-Gal-glycosylation at N-linkage in expressed protein. Briefly, the cells were grown in Dulbecco's modified eagle medium (DMEM, Lonza) containing 10% fetal bovine serum (FBS, Gibco), 100 µg/mL penicillin, 100 µg/mL streptomycin, 100 µg/mL geneticin (Sigma Aldrich) and 4 mM L-glutamine (Gibco), hereafter called DMEMc, at 37°C, 95% humidity and 5% CO_2 fumigation. Transfection of the cells was carried out with Lipofectamine 2000 (Invitrogen) according to the recommendations of the manufacturer. Stably transfected cells were grown in a petri dish in DMEMc at 37°C with 95% humidity and 5% CO_2 and selected by addition of zeocin (250 µg/ml); single clones were picked after 5-6 days and cultured in 96-well cell-culture plates (Greiner bio-one). For clonal selection, protein expression in supernatant was analysed in sandwich ELISA (detailed description below) using anti-V5 antibody coating and anti-His-biotin as secondary antibody for detection. Clones expressing AP-N were propagated into 6-well culture plates and upon reaching 80-90% confluency, cells were suspended in recovery™ cell culture freezing medium (Gibco) and stored in liquid nitrogen for future use. For generation of stable cell-lines, clones were further propagated to T-25, T-75, and T-175 flasks (Greiner Bio-One) in DMEMc and zeocin. From T-175 flasks, freeze stocks were prepared and clones were used for large scale production.

For protein production, stably transfected single clones (in HEK 293-GT) were mass produced in cell-stack (CELLdisc, Greiner bio-one) with a working volume of 500 ml using complete media (DMEMc) supplemented with zeocin (50 µg/ml) and allowed to grow until 70-80% confluent. Thereafter cells were gently washed with pre-warmed PBS and serum-free media was added (Gibco FreeStyle 293) and incubated for 48 hours at 37°C, 95% humidity and 5% CO_2. The supernatant with expressed protein was collected and complete media (DMEMc) added to the cells followed by a 24 hour incubation period, thereafter cells were washed and a second round of production in serum free media was carried out. Supernatant was collected, filtered on 0.22 µm vacuum filter (VWR®) and stored at -20°C until purification.

Proteins were purified using immobilized metal affinity chromatography (IMAC) chromatography (NGC Chromatography, Biorad). Briefly, supernatant was applied onto a 5ml HisTrap column (HisTrap HP, GE Healthcare) and 20 mM sodium phosphate buffer with 0.5 M NaCl and 20 mM imidazole was used as binding and washing buffers. Bound proteins were eluted with 20 mM sodium phosphate, 0.5 M NaCl (pH 7.4) buffer with a gradient concentration of 0-500 mM imidazole. The eluted protein fractions were analysed by ELISA, pooled and buffer exchanged with PBS using centrifugal filter units (cutoff 3 kD) with cellulose membrane and as per manufacturer's instructions (Amicon Ultra 15, Merck Millipore), concentrated protein was stored at -20°C. Protein quantity was determined on nanodrop and by using Bradford's assay, and protein quality was analysed in SDS-PAGE, immunoblot and ELISA as per established standard laboratory protocols.

Sandwich ELISA screening

AP-N proteins were designed to contain a C-terminal V5 tag for protein detection and a histidine (6x) tag to assist in protein purification. AP-N expression in cell-culture supernatant as well as its detection in purified HisTrap fractions obtained in chromatography (IMAC) were analysed in sandwich ELISA using anti-V5 (Invitrogen R96025) and anti-His-biotin (Invitrogen MA1-21315-BTIN) antibodies. Briefly, for detection of AP-N protein ELISA micro-well plates (Nunc) were coated overnight with 100 µl/well of anti-V5 antibody (2 µg/ml) in PBS and next day washed 4x with TBST (Tris-buffer saline supplemented with 0.05% Tween-20) and blocked with 300 µl of 3% BSA/TBST for 1 hr/rt (hour at room temperature). Thereafter, 100 µl of supernatant/HisTrap fraction was added to the wells and incubated for 2hr/rt. After washing the wells secondary antibody was added (anti-His-biotin, 1:2000 in 3% BSA/TBST) for 90 minutes followed by incubation with streptavidin conjugated with alkaline phosphatase (BD biosciences BD 554065; 1:1000 in 3% BSA/TBST) for 45 minutes. Signal was developed with pNPP (p-NitroPhenyl Phosphate, Carl Roth) as substrate and read at 405 nm.

SDS-PAGE

MiniPROTEAN TGX precast gels (AnyKD™, Biorad) were used for protein analysis; 5 µg recombinant protein mixed with loading buffer (Laemmli sample buffer with β-mercaptoethanol) was heated for 5 mins at 95°C and loaded into wells (volume 20-30 µl/well). Thereafter, proteins were resolved by running for 10 mins at 100V followed by 30-40 minutes at 200V [16]. For visualization by staining – Coomassie blue, silver stain, or Sypro-Ruby were used as per manufacturer's instructions.

Immunoblot

For immunoblot, proteins run on precast gels were electrophoretically transferred onto PVDF (polyvinylidene difluoride, Thermo Fischer Scientific) membrane and blocked with 3% BSA/TBST followed by incubation with anti-APN-IgG (1:5000 in 3% BSA/TBST), obtained from polyclonal rabbit serum by immunizing rabbit with recombinant AP-N (Eurogentec), for AP-N detection. Anti-α-Gal IgM (M86, Enzo lifesciences; 1:10 in 3% BSA/TBST) was used for α-Gal detection. Anti-rabbit-IgG or anti-mouse-IgM (Southern biotech; 1:1000 in 3% BSA/TBST) conjugated to alkaline phosphatase (AP) were used as secondary antibodies and signal developed with NBT/BCIP (nitro-blue tetrazolium and 5-bromo-4-chloro-3'-indolyphosphate) substrate solution (ThermoFischer Scientific).

AP-N characterization

AP-N activity assay – AP-N enzymatic activity was determined at 10 nM enzyme concentration in 200 µl 50 mM Tris-buffer at pH 7, with L-leucine-p-nitroanilide (Sigma-Aldrich) as substrate in gradient concentration from 100 µM to 10 mM. AP-N and substrate were incubated at 37°C for 90 minutes. Formation of product p-nitroanilide was measured in a spectrophotometer (Nanodrop) at 405 nm [29].

Basophil activation test (BAT) – The Flow CAST® (BüHLMANN Laboratories AG) assay was used for quantitative measurement of *in vitro* basophil activation using venous blood from an α-Gal allergic patient, the assay was performed within 24 hours of blood collection using increasing concentrations of the mutant protein, as described before (please refer chapter III) [16]. Anti-FcεRI and fMLP are standard positive controls and BAT assay is considered valid if the controls reach minimum 10% CD63 upregulation.

Proteomics analysis – performed by the proteomics facility as per their standard established procedure, proteins were annotated using human and porcine database.

RESULTS

4.1 Mutant modelling

Chen et al. reported the structure of AP-N in 2012 by crystalizing porcine AP-N ectodomain [29]. Figure 2 shows the sequence of porcine aminopeptidase N (PDB id: 4FKK; Uniprot P15145). AP-N is 963 amino acids long and has a molecular weight of 109 kDa (theoretical weight), it contains 14 potential N-linked (N-X-S/T) glycosylation sites (figure 2) out of which 13 sites are glycosylated in the reported structure (Asn736 not glycosylated) [29]. The protein crystal structure (resolution 1.85 Å) was visualized in PyMol, the glycosylations sites to be mutated were selected based on their relative position, and three mutants were designed (figure 3). Three glycosylations were left intact in each mutant and the positions were determined based upon distance of glycosylated residues on the AP-N structure as seen in the space-filled model. These mutants represent three distinct structures to study significance of epitope proximity in the context of a carbohydrate (α-Gal) allergen, and namely represent three possible models in term of spatial distribution – epitopes close together (between 20 to 40 Å), epitopes on opposite sites (>40 Å) on the protein, and epitopes at an intermediate distance (≥40 Å) (figure 3).

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SEQUENCE 963 AA; 109 kDa

        10         20         30         40         50         60
MAKGFYISKA¦LGILGILLGV¦AAVATIIALS¦VVYAQEKNKN¦AEHVPQAPTS¦PTITTTAAIT
        70         80         90        100        110        120
LDQSKPWNRY¦RLPTTLLPDS¦YNVTLRPYLT¦PNADGLYIFK¦GKSIVRLLCQ¦EPTDVIIIHS
       130        140        150        160        170        180
KKLNYTTQGH¦MVVLRGVGDS¦QVPEIDRTEL¦VELTEYLVVH¦LKGSLQPGHM¦YEMESEFQGE
       190        200        210        220        230        240
LADDLAGFYR¦SEYMEGNVKK¦VLATTQMQST¦DARKSFPCFD¦EPAMKATFNI TLIHPNNLTA
       250        260        270        280        290        300
LSNMPPKGSS¦TPLAEDPNWS¦VTEFETTPVM¦STYLLAYIVS¦EFQSVNETAQ¦NGVLIRIWAR
       310        320        330        340        350        360
PNAIAEGHGM¦YALNVTGPIL¦NFFANHYNTS¦YPLPKSDQIA¦LPDFNAGAME¦NWGLVTYREN
       370        380        390        400        410        420
ALLFDPQSSS¦ISNKERVVTV¦IAHELAHQWF¦GNLVTLAWWN¦DLWLNEGFAS¦YVEYLGADHA
       430        440        450        460        470        480
EPTWNLKDLI¦VPGDVYRVMA¦VDALASSHPL¦TTPAEEVNTP¦AQISEMFDSI¦SYSKGASVIR
       490        500        510        520        530        540
MLSNFLTEDL¦FKEGLASYLH¦AFAYQNTTYL¦DLWEHLQKAV¦DAQTSIRLPD¦TVRAIMDRWT
       550        560        570        580        590        600
LQMGFPVITV¦DTKTGNISQK¦HFLLDSESNV TRSSAFDYLW¦IVPISSIKNG¦VMQDHYWLRD
       610        620        630        640        650        660
VSQAQNDLFK¦TASDDWVLLN¦VNVTGYFQVN¦YDEDNWRMIQ¦HQLQTNLSVI¦PVINRAQVIY
       670        680        690        700        710        720
DSFNLATAHM¦VPVTLALDNT¦LFLNGEKEYM¦PWQAALSSLS¦YFSLMFDRSE¦VYGPMKKYLR
       730        740        750        760        770        780
KQVEPLFQHF¦ETLTKNWTER¦PENLMDQYSE¦INAISTACSN¦GLPQCENLAK¦TLFDQWMSDP
       790        800        810        820        830        840
ENNPIHPNLR¦STIYCNAIAQ¦GGQDQWDFAW¦GQLQQAQLVN¦EADKLRSALA¦CSNEVWLLNR
       850        860        870        880        890        900
YLGYTLNPDL¦IRKQDATSTI¦NSIASNVIGQ¦PLAWDFVQSN¦WKKLFQDYGG¦GSFSFSNLIQ
       910        920        930        940        950        960
GVTRRFSSEF¦ELQQLEQFKK¦NNMDVGFGSG¦TRALEQALEK¦TKANIKWVKE¦NKEVVLNWFI

EHS
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Figure 2. **Aminopeptidase N (AP-N) sequence** retrieved from the Protein Data Bank (PDB id: 4FKK) – the N-linked glycosylation sites are attached to the protein on the asparagine residue in a consensus sequence represented as Asn-X-Ser/Thr (X can be any amino acid except proline). Fasta sequence of the porcine AP-N is shown here (only ectodomain - residue 33 – 963 was used for mutagenesis and protein expression); brown residues show the potential N-linkage glycosylation sites present on the AP-N structure. There are 14 potential sites present for glycan linkage.

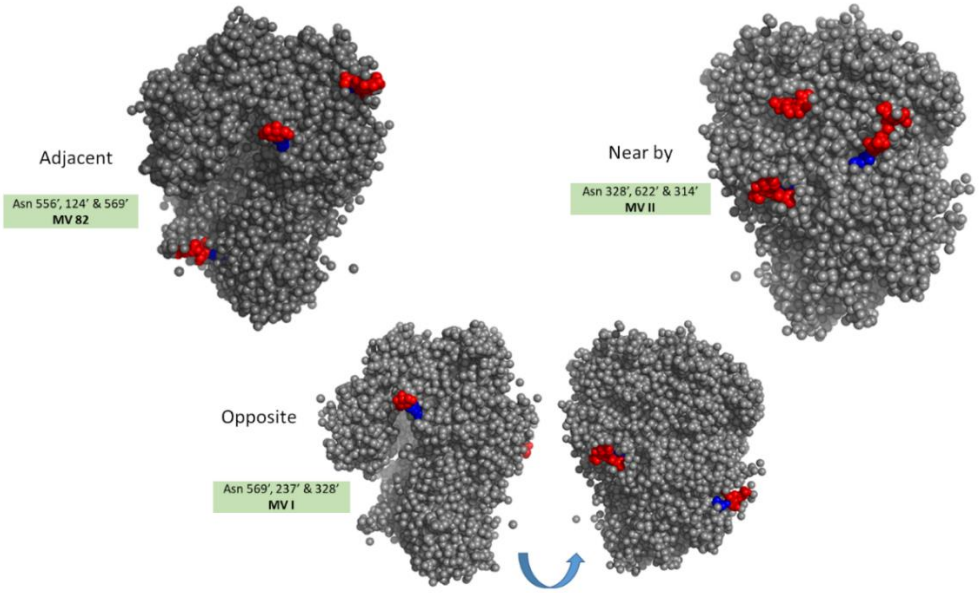

Figure 3. **Aminopeptidase N mutant modelling** – space-filled model of the porcine aminopeptidase N (PDB id: 4FKK) showing glycosylation (in red) on the asparagine (blue) residue. Mutant model as visualized in PyMol show the spatial arrangement of three glycosylation sites after elimination of remaining 11 glycosylation sites. Arrangement of the glycosylation in the three mutants is shown – adjacent (MV 82), near by (MV II), and opposite (MV I). The green rectangles give the un-mutated asparagine (Asn) position within the AP-N sequence.

For generation of mutants, AP-N ectodomain (aa 33-963) was inserted into the vector (pBudCE 4.1) as described in the methods section. I planned to introduce 2-3 mutations per cycle in order to increase the chances of successful mutation. Figure 4 shows a step-wise mutagenesis plan, Asn (asparagine) residue is followed by its position (no.) as numbered in the AP-N sequence (PDB) fasta file. The Asn (genetic code: AAT/AAC) residue is mutated to Ala (alanine, genetic code: GCC/GCT/GCG/GCA) to remove N-linkage site. First six mutations are basic mutations and they are common to all three final structures (mutants). Firstly, basic mutations (figure 4, orange box) were introduced in the parent AP-N clone to obtain an intermediate clone with eight remaining sites (43% sites mutated); these clones were transfected into HEK 293-GT cells and expressed to check protein production and stability. Thereafter specific mutations were introduced sequentially in the intermediate clone to arrive at the final mutants with glycosylation at three specific positions (figure 3). Flowchart showing planning of the introduction of mutation and mutant generation is depicted in figure 4. Each box represents one PCR cycle and 'Asn' followed by residue location, within the sequence, represents the individual sites that were mutated. Green oval boxes show glycosylation configuration of the final mutants.

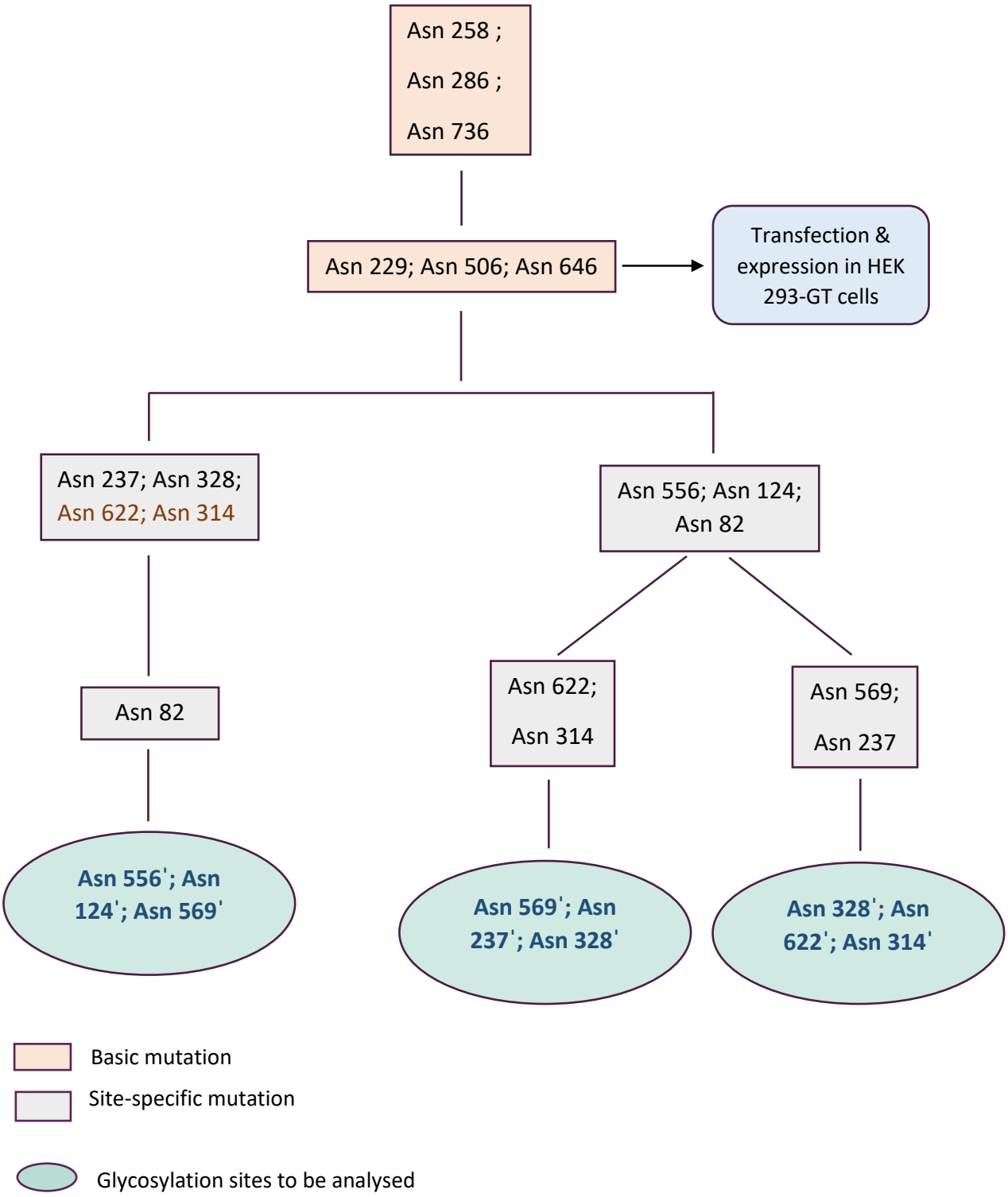

Figure 4. Flowchart depicting sequential mutation plan of the porcine aminopeptidase N. Asn (asparagine) is followed by its position within the AP-N sequence. Green oval shows final mutants with intact glycosylation sites.

4.2 Site-directed mutagenesis and mutant construction

For site-directed mutagenesis, APN gene (ectodomain, aa 33-963) was inserted into the pBudCE4.1 vector (4.6 kb, Invitrogen V532-20), this vector contains a C-terminal peptide encoding V5 epitope for protein detection and a polyhistidine (6xHis) tag for detection as well as purification of the recombinant proteins (figure 5). In addition, zeocin resistance gene allows for clone selection in *E.coli* and creation of stable mammalian cell lines.

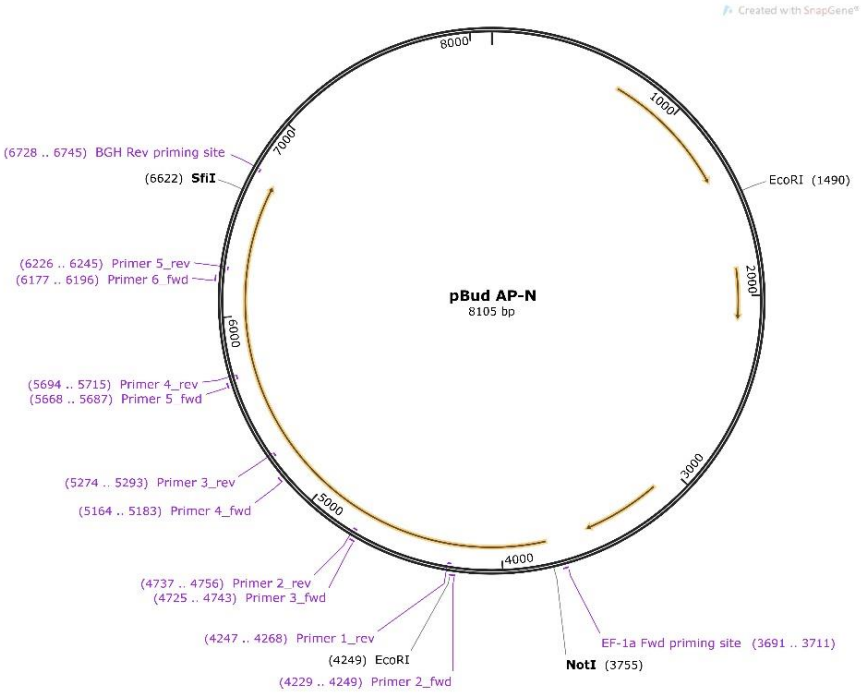

Figure 5. **pBudCE4.1 vector map**: APN gene inserted into the vector showing primer sequences used in analyzing AP-N mutants.

Mutagenesis: The final mutant clones (green oval, figure 4) were obtained after eight rounds of mutagenesis cycles and at least 2 positive clones i.e. clones with successful mutations were obtained at each stage. Higher number of positive clones were obtained during early mutation cycles (PCR cycle 1-2, figure 4) compared to those performed at a later stage (PCR cycle 3-4, figure 4). Mutation efficiency was monitored with control mutagenesis reaction on pUC18 control plasmid (QuikChange Multi Site-directed mutagenesis kit, Agilent) with mutated (inactive, 3 sites were mutated) *LacZ* gene which was back-mutated (3 primer templates) to get an active *LacZ* gene. Active *LacZ* gene leads to production of blue *E.coli* colonies upon successful mutation due to the expression of β-galactosidase enzyme by active *LacZ* gene (see methods section for details on blue-white screening). Overall >50% efficiency was observed in the control mutagenesis reaction.

4.3 Analysis of mutant sequences

Mutated clones were analyzed by sequencing in a cycle sequencer using Big Dye labelling. The mutants were screened for successful introduction of mutation by sequence alignment. Figure 6 shows a representative sequence alignment of mutants (F1/B11/C9) and parent AP-N. Sequences here show

mutation in clones selected for protein expression, F1 is an intermediate clone obtained after mutating 6 residues whereas C9 (MVII, residues 'near by') and B11 (MV I, residues 'opposite') were final mutants, the clone with residues at intermediate distance (MV82) was removed later from analysis due to difficulty in obtaining a stable cell line and hence is not shown here in sequence alignment.

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AP-N      MAKGFYISKALGILGILLGVAAVATIIALSVVYAQEKNKNAEHVPQAPTSPTITTTAAIT   60
F1        MAKGFYISKALGILGILLGVAAVATIIALSVVYAQEKNKNAEHVPQAPTSPTITTTAAIT   60
C9        MAKGFYISKALGILGILLGVAAVATIIALSVVYAQEKNKNAEHVPQAPTSPTITTTAAIT   60
B11       MAKGFYISKALGILGILLGVAAVATIIALSVVYAQEKNKNAEHVPQAPTSPTITTTAAIT   60
          ************************************************************

AP-N      LDQSKPWNRYRLPTTLLPDSYNVTLRPYLTPNADGLYIFKGKSIVRLLCQEPTDVIIIHS   120
F1        LDQSKPWNRYRLPTTLLPDSYNVTLRPYLTPNADGLYIFKGKSIVRLLCQEPTDVIIIHS   120
C9        LDQSKPWNRYRLPTTLLPDSYAVTLRPYLTPNADGLYIFKGKSIVRLLCQEPTDVIIIHS   120
B11       LDQSKPWNRYRLPTTLLPDSYAVTLRPYLTPNADGLYIFKGKSIVRLLCQEPTDVIIIHS   120
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AP-N      KKLNYTTQGHMVVLRGVGDSQVPEIDRTELVELTEYLVVHLKGSLQPGHMYEMESEFQGE   180
F1        KKLNYTTQGHMVVLRGVGDSQVPEIDRTELVELTEYLVVHLKGSLQPGHMYEMESEFQGE   180
C9        KKLAYTTQGHMVVLRGVGDSQVPEIDRTELVELTEYLVVHLKGSLQPGHMYEMESEFQGE   180
B11       KKLAYTTQGHMVVLRGVGDSQVPEIDRTELVELTEYLVVHLKGSLQPGHMYEMESEFQGE   180
          *** ************************************************************

AP-N      LADDLAGFYRSEYMEGNVKKVLATTQMQSTDARKSFPCFDEPAMKATFNITLIHPNNLTA   240
F1        LADDLAGFYRSEYMEGNVKKVLATTQMQSTDARKSFPCFDEPAMKATFAITLIHPNNLTA   240
C9        LADDLAGFYRSEYMEGNVKKVLATTQMQSTDARKSFPCFDEPAMKATFAITLIHPNALTA   240
B11       LADDLAGFYRSEYMEGNVKKVLATTQMQSTDARKSFPCFDEPAMKATFAITLIHPNNLTA   240
          ********************************************* ******* ***

AP-N      LSNMPPKGSSTPLAEDPNWSVTEFETTPVMSTYLLAYIVSEFQSVNETAQNGVLIRIWAR   300
F1        LSNMPPKGSSTPLAEDPAWSVTEFETTPVMSTYLLAYIVSEFQSVAETAQNGVLIRIWAR   300
C9        LSNMPPKGSSTPLAEDPAWSVTEFETTPVMSTYLLAYIVSEFQSVAETAQNGVLIRIWAR   300
B11       LSNMPPKGSSTPLAEDPAWSVTEFETTPVMSTYLLAYIVSEFQSVAETAQNGVLIRIWAR   300
          ***************** ************************** **************

AP-N      PNAIAEGHGMYALNVTGPILNFFANHYNTSYPLPKSDQIALPDFNAGAMENWGLVTYREN   360
F1        PNAIAEGHGMYALNVTGPILNFFANHYNTSYPLPKSDQIALPDFNAGAMENWGLVTYREN   360
C9        PNAIAEGHGMYALNVTGPILNFFANHYNTSYPLPKSDQIALPDFNAGAMENWGLVTYREN   360
B11       PNAIAEGHGMYALAVTGPILNFFANHYNTSYPLPKSDQIALPDFNAGAMENWGLVTYREN   360
          ************* ************************************************

AP-N      ALLFDPQSSSISNKERVVTVIAHELAHQWFGNLVTLAWWNDLWLNEGFASYVEYLGADHA   420
F1        ALLFDPQSSSISNKERVVTVIAHELAHQWFGNLVTLAWWNDLWLNEGFASYVEYLGADHA   420
C9        ALLFDPQSSSISNKERVVTVIAHELAHQWFGNLVTLAWWNDLWLNEGFASYVEYLGADHA   420
B11       ALLFDPQSSSISNKERVVTVIAHELAHQWFGNLVTLAWWNDLWLNEGFASYVEYLGADHA   420
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AP-N      EPTWNLKDLIVPGDVYRVMAVDALASSHPLTTPAEEVNTPAQISEMFDSISYSKGASVIR   480
F1        EPTWNLKDLIVPGDVYRVMAVDALASSHPLTTPAEEVNTPAQISEMFDSISYSKGASVIR   480
C9        EPTWNLKDLIVPGDVYRVMAVDALASSHPLTTPAEEVNTPAQISEMFDSISYSKGASVIR   480
B11       EPTWNLKDLIVPGDVYRVMAVDALASSHPLTTPAEEVNTPAQISEMFDSISYSKGASVIR   480
          ************************************************************

AP-N      MLSNFLTEDLFKEGLASYLHAFAYQNTTYLDLWEHLQKAVDAQTSIRLPDTVRAIMDRWT   540
F1        MLSNFLTEDLFKEGLASYLHAFAYQATTYLDLWEHLQKAVDAQTSIRLPDTVRAIMDRWT   540
C9        MLSNFLTEDLFKEGLASYLHAFAYQATTYLDLWEHLQKAVDAQTSIRLPDTVRAIMDRWT   540
B11       MLSNFLTEDLFKEGLASYLHAFAYQATTYLDLWEHLQKAVDAQTSIRLPDTVRAIMDRWT   540
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AP-N      LQMGFPVITVDTKTGNISQKHFLLDSESNVTRSSAFDYLWIVPISSIKNGVMQDHYWLRD   600
F1        LQMGFPVITVDTKTGNISQKHFLLDSESNVTRSSAFDYLWIVPISSIKNGVMQDHYWLRD   600
C9        LQMGFPVITVDTKTGAISQKHFLLDSESAVTRSSAFDYLWIVPISSIKNGVMQDHYWLRD   600
B11       LQMGFPVITVDTKTGAISQKHFLLDSESNVTRSSAFDYLWIVPISSIKNGVMQDHYWLRD   600
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AP-N    VSQAQNDLFKTASDDWVLLNVNVTGYFQVNYDEDNWRMIQHQLQTNLSVIPVINRAQVIY   660
F1      VSQAQNDLFKTASDDWVLLNVNVTGYFQVNYDEDNWRMIQHQLQTALSVIPVINRAQVIY   660
C9      VSQAQNDLFKTASDDWVLLNVNVTGYFQVNYDEDNWRMIQHQLQTALSVIPVINRAQVIY   660
B11     VSQAQNDLFKTASDDWVLLNVAVTGYFQVNYDEDNWRMIQHQLQTALSVIPVINRAQVIY   660
        ********************.**********************.**************

AP-N    DSFNLATAHMVPVTLALDNTLFLNGEKEYMPWQAALSSLSYFSLMFDRSEVYGPMKKYLR   720
F1      DSFNLATAHMVPVTLALDNTLFLNGEKEYMPWQAALSSLSYFSLMFDRSEVYGPMKKYLR   720
C9      DSFNLATAHMVPVTLALDNTLFLNGEKEYMPWQAALSSLSYFSLMFDRSEVYGPMKKYLR   720
B11     DSFNLATAHMVPVTLALDNTLFLNGEKEYMPWQAALSSLSYFSLMFDRSEVYGPMKKYLR   720
        ************************************************************

AP-N    KQVEPLFQHFETLTKNWTERPENLMDQYSEINAISTACSNGLPQCENLAKTLFDQWMSDP   780
F1      KQVEPLFQHFETLTKAWTERPENLMDQYSEINAISTACSNGLPQCENLAKTLFDQWMSDP   780
C9      KQVEPLFQHFETLTKAWTERPENLMDQYSEINAISTACSNGLPQCENLAKTLFDQWMSDP   780
B11     KQVEPLFQHFETLTKAWTERPENLMDQYSEINAISTACSNGLPQCENLAKTLFDQWMSDP   780
        ***************.********************************************

AP-N    ENNPIHPNLRSTIYCNAIAQGGQDQWDFAWGQLQQAQLVNEADKLRSALACSNEVWLLNR   840
F1      ENNPIHPNLRSTIYCNAIAQGGQDQWDFAWGQLQQAQLVNEADKLRSALACSNEVWLLNR   840
C9      ENNPIHPNLRSTIYCNAIAQGGQDQWDFAWGQLQQAQLVNEADKLRSALACSNEVWLLNR   840
B11     ENNPIHPNLRSTIYCNAIAQGGQDQWDFAWGQLQQAQLVNEADKLRSALACSNEVWLLNR   840
        ************************************************************

AP-N    YLGYTLNPDLIRKQDATSTINSIASNVIGQPLAWDFVQSNWKKLFQDYGGGSFSFSNLIQ   900
F1      YLGYTLNPDLIRKQDATSTINSIASNVIGQPLAWDFVQSNWKKLFQDYGGGSFSFSNLIQ   900
C9      YLGYTLNPDLIRKQDATSTINSIASNVIGQPLAWDFVQSNWKKLFQDYGGGSFSFSNLIQ   900
B11     YLGYTLNPDLIRKQDATSTINSIASNVIGQPLAWDFVQSNWKKLFQDYGGGSFSFSNLIQ   900
        ************************************************************

AP-N    GVTRRFSSEFELQQLEQFKKNNMDVGFGSGTRALEQALEKTKANIKWVKENKEVVLNWFI   960
F1      GVTRRFSSEFELQQLEQFKKNNMDVGFGSGTRALEQALEKTKANIKWVKENKEVVLNWFI   960
C9      GVTRRFSSEFELQQLEQFKKNNMDVGFGSGTRALEQALEKTKANIKWVKENKEVVLNWFI   960
B11     GVTRRFSSEFELQQLEQFKKNNMDVGFGSGTRALEQALEKTKANIKWVKENKEVVLNWFI   960
        ************************************************************

AP-N    EHS   963
F1      EHS   963
C9      EHS   963
B11     EHS   963
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Figure 6. Fasta sequence alignment of the mutants F1, B11, and C9 showing mutated residues: asparagine (genetic code: AAT/AAC) is replaced with alanine (genetic code: GCC/GCT/GCG/GCA) at 6 N-linkage positons for clone F1 and 11 N-linkage positions for clones C9 and B11. Red – mutations in F1, C9 and B11, blue – mutations in C9 and B11, green – mutations unique to C9, purple – mutations unique to B11. N-linkage sites in parent sequence are (AP-N) underlined; yellow highlight shows un-mutated sites in F1, C9 and B11.

4.4 Mutant screening for protein production

For clonal production of the mutated AP-N protein, mutant plasmids were transfected into HEK 293-GT cells (HEK cells with α-1,3-galactosyltransferase activity) using lipofectamine. After transfection cells were grown in a petri dish for 3-4 days. Thereafter single clones were picked and grown in 96 well cell-culture plates with zeocin (250 µg/ml) selection pressure, after 4-5 days surviving clones were transferred to a 6-well cell culture plates for expansion, and supernatant was collected when cells were 90% confluent. Presence of expressed protein in supernatant was detected in sandwich ELISA using antibodies directed at the V5 and His Tags (see methods section for details). Several screenings were carried out and figure 7 shows results from one representative screening ELISA, clone B is a mutant with 3 glycosylations and residues on opposite ends (MV I), clones C and D have 3 glycosylations close together (MV II), clones E and F are intermediate clones (6 sites mutated) and G and H are mutants

with 3 glycosylations at residues adjacent to each other (MV82). Positive clones were identified for all four mutants and stable cell lines were created and freeze stocks prepared for future use. Of note, only three mutants were analyzed further. Mutant with 'adjacent' residues (clones G3 and H3, figure 7) was unstable and hence excluded.

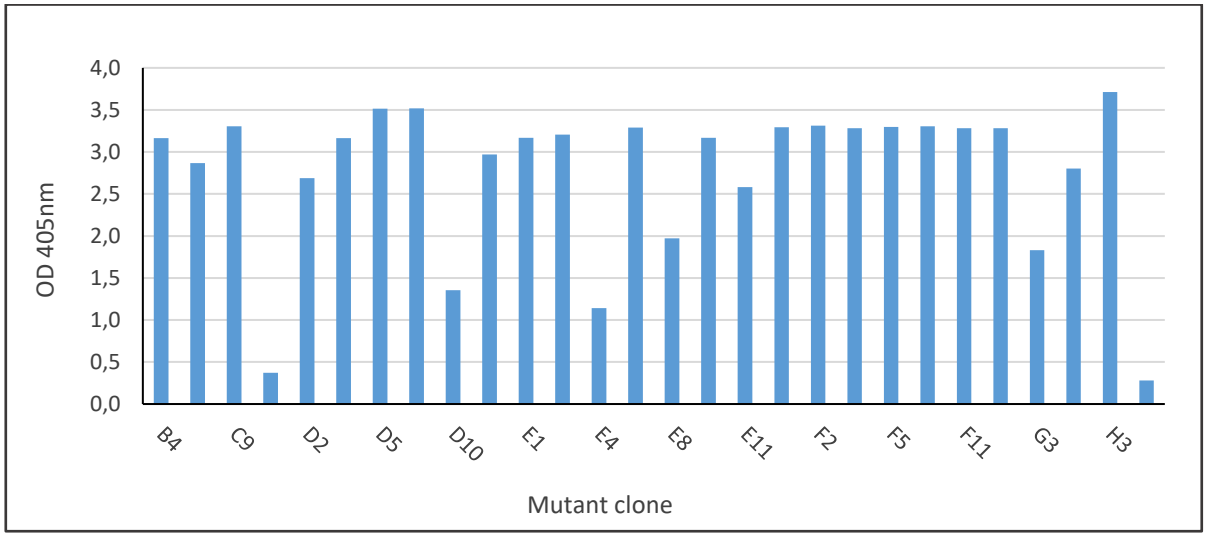


Figure 7. **ELISA detection to screen clones expressing mutant AP-N**: Sandwich ELISA using anti-V5 coating and anti-His-biotinylated secondary antibody to detect AP-N expression in the supernatant. Clones – B is mutant with 3 glycosylations and residues on opposite ends (MV I), C and D have 3 glycosylations close together (MV II), E and F are intermediate clones (6 sites mutated) and G and H are mutants with 3 glycosylations at residues adjacent to each other (MV82). X-axis: clone name, y-axis: optical density at 405 nm.

Aminopeptidase N (AP-N)

To aid in protein analysis and characterization of mutants, parent AP-N were produced to use as control. For production, stable cell lines expressing AP-N in HEK 293 cells with (APN+) and without (APN-) α-1,3-GT activity were expanded and both type of AP-N proteins (with/without α-Gal) were successfully expressed in the serum free media (figure 8). Molecular weight of both AP-N proteins is higher than predicted weight (109 kD) due to the presence of glycosylation as well as additional inserted tags (V5 and 6xHis) to assist with screening and purification.

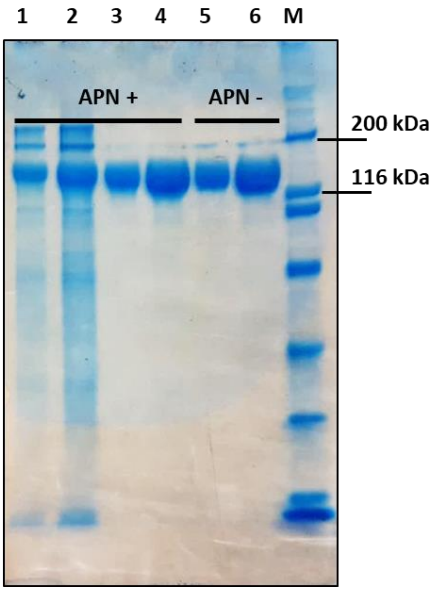


Figure 8. **Expression of aminopeptidase N (AP-N)**: expression of original non-mutated AP-N clone in both HEK 293 (without α-1,3-GT activity) and HEK 293-GT (with α-1,3-GT activity) cells. Coomassie blue staining; lanes 1-4 show HisTrap purified AP-N fractions with α-Gal glycosylation (APN+) and lanes 5 and 6 show pooled APN fractions without α-Gal glycosylation. Marker (M) – broad range, Biorad.

4.5 Mutant protein expression and purification

Stable cell lines of each of the mutant clones F1, C9 and B11 were grown in three T-175 cell culture flasks (Greiner bio-one) with DMEMc and zeocin (100 µg/ml) and upon reaching 90% confluency they were transferred to cell stack (CELLdisc, Greiner bio-one) with a capacity of 500 ml for mass production of proteins. Cells were grown in complete media (DMEMc with zeocin) until they were 70-80% confluent, thereafter the media was removed and cells were gently washed with pre-warmed PBS and 500 ml of serum-free media (FreeStyle 293, Gibco) was added. Cells were allowed to produce for 48 hours and supernatant was collected; cells were again fed with complete media for 24 hours followed by a second round of production in serum-free media for another 48 hours. Supernatants (spent FreeStyle 293) were combined, filtered on 0.22 µm filters (Amicon) and purified on a 5 ml HisTrap (GE Healthcare) column *via* IMAC chromatography.

Eluted proteins were collected in 1.5 ml tubes in several fractions hereafter called as HisTrap fractions, 150 µl aliquot from each fractions was drawn for analysis. Aliquots were analysed for detection of mutant APN in sandwich ELISA as previously described and in silver stain gel to detect presence of non-specific proteins. Thereafter, HisTrap fractions were pooled and buffer exchanged with PBS on centrifugal filter tubes made of cellulose membrane with a cutoff of 3 kD (Amicon Ultra-15, Merck Millipore). Pooled fractions were exchanged with PBS 3x by centrifuging at 4000xg for 15mins/cycle. The proteins were stored at -20°C with 10% glycerol.

Clone F1

F1 clone of the porcine aminopeptidase contains 6 mutations (asparagine to alanine) therefore exhibiting possibility of glycosylation on 8 sites (N-X-S/T) instead of 14 as in the original structure. The mutant clone was expressed in serum free medium and purified by IMAC chromatography (NGC Chromatography, Biorad) on a 5 ml HisTrap column. The mutant protein is expressed with 6x histidine

tag which enables its binding to the column and the bound protein is then eluted by addition of imidazole at increasing concentrations. Eluted HisTrap fractions were analyzed in sandwich ELISA (figure 9A, left) to detect mutant AP-N (F1). Several fractions with mutant AP-N protein are detected in ELISA screening; sliver stain (figure 9A, right) shows presence of co-eluted proteins in the fractions A5 – A7 and no/low detection in fractions A36 – A37 whereas A26 and A27 contain mutant protein (blue dotted box) along with some high molecular non-specific proteins. Based upon signal detected in the sandwich ELISA fractions (showing AP-N expression) were selected and pooled for use in the next step of analysis. Fractions A24 to A28 were pooled together (PI) and fractions A34 to A39 were pooled together (PII) and concentrated.

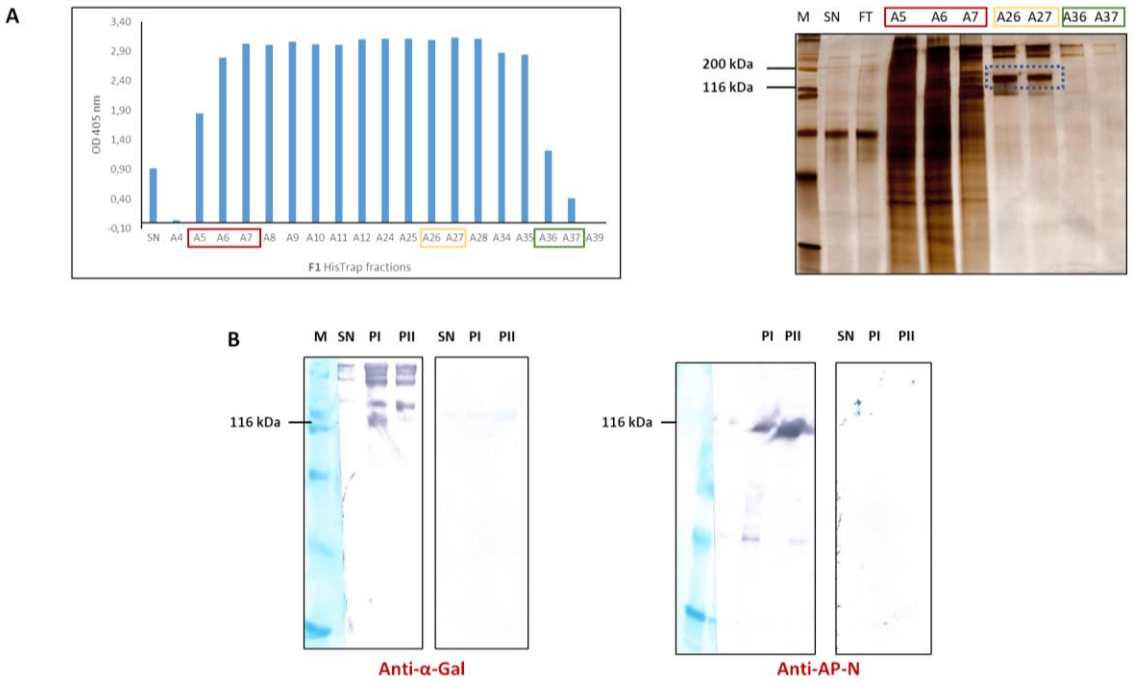


Figure 9. Characterization of F1 mutant protein produced in HEK 293-GT cells by ELISA, silver stain, and immunoblot: A. AP-N protein obtained after HisTrap purification (ELISA detection of eluted HisTrap fractions, left), proteins of selected fractions visualized in silver stain (right). B. detection of AP-N (right) and α-Gal (left) by immunoblot.

The concentrated F1 (PI and PII) protein pools were next analyzed in immunoblot to confirm the presence of AP-N using a rabbit anti-AP-N polyclonal serum (Figure 9B, right) and presence of α-Gal using anti-α-Gal IgM (figure 9B, left). The 116 kDa band is identified as mutant AP-N protein by anti-AP-N antibody detection (right), successful expression of α-Gal-containing glycosylation (left) is seen *via* anti-α-Gal staining. Anti-α-Gal revealed additional bands at high molecular weight that were not visible using the anti-AP-N antibody.

Clones C9 (MV II) and B11 (MV I)

Mutant proteins expressed from clones C9 and B11 were analyzed in a similar way. Figure 10 shows an analysis of HisTrap fractions by ELISA and by silver staining. Proteins were produced in serum free media and purified and analysed as described for F1, Eluted fractions were screened for AP-N expression by sandwich ELISA (anti-V5/anti-His-biotin) and fractions showing higher protein quantities

were resolved on SDS-PAGE gel (anyKD, Biorad) and silver stained. C9 protein was eluted in 2 peaks (figure 10A, left) at an imidazole concentration of 150 mM and 250 mM and mutant proteins were detected in fractions B19 – B41 and C1 – C13. Silver staining of fractions with high ELISA signal (figure 10A, right) revealed the presence of multiple proteins which were co-eluted during the purification step. Molecular weight of AP-N (with glycosylation) is approximately 120 kDa, proteins detected above 200 kDa could not be identified and perhaps occur due to the formation of protein aggregates.

B11 protein was eluted in 3 peaks (figure 10B, left- red, green, and yellow box) at 25 mM, 50 mM, and 200 mM concentration of imidazole. Fractions from all three chromatographic purification and elution gradients were pooled in three pools (PI, PII, PIII) and buffer exchanged with PBS and concentrated. The silver stain gel of the pools PI, PII and PIII (figure 10B, right) reveals high molecular weight bands which are >200 kDa in size and possibly represent aggregate formation/non-specific proteins in the eluted fractions.

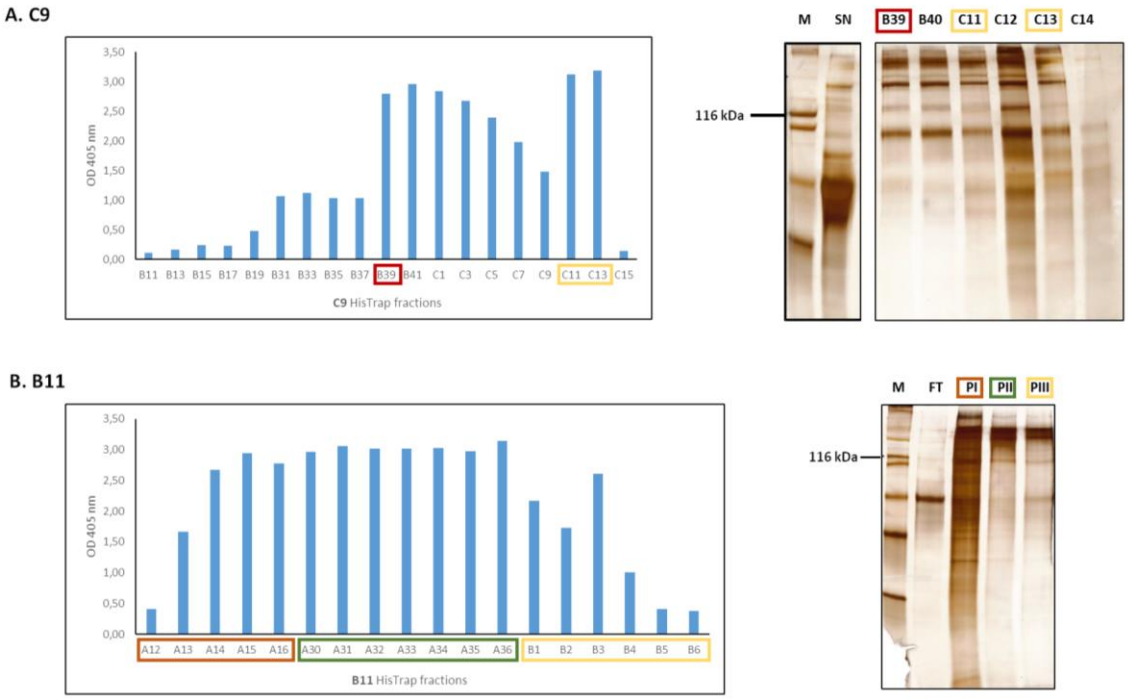


Figure 10. **Characterization of proteins produced by mutant clones B11 and C9 in ELISA and silver stain gel**: HisTrap fractions obtained after IMAC purification analyzed in sandwich ELISA to detect AP-N protein, left (A, clone C9; B, clone B11). Fractions separated on gel and silver stained to visualize presence of co-eluted non-specific proteins, right (A, C9; B, B11). M - marker (broad range, Biorad), SN – supernatant, FT-flow through, P I/II/III – pool I/II/III. ELISA – x-axis – HisTrap fraction number, y-axis – optical density at 405 nm.

The pooled fractions of clones B11 and C9 were next analyzed in immunoblot to confirm the identity of mutant AP-N protein using anti-APN-IgG antibody (figure 11A, left), and using anti-α-Gal-IgM (M86) antibody (figure 11B) to detect presence of α-Gal glycosylation onto the expressed protein. No AP-N reactive bands could be detected in proteins purified from clones B11 and C9 whereas recombinant AP-N was successfully detected in the positive control (non-mutated AP-N protein) as well as in proteins produced by clone F1 (6 sites mutated to alanine). Next, fractions were analyzed by silver stain to visualize the protein content. Proteins of high molecular weight were present in all mutants (F1, B11 and C9) whereas no high molecular weight bands were seen in the non-mutated recombinant

AP-N. For detection of α-Gal epitopes on mutant proteins, a second immunoblot of C9 and B11 was performed and glycosylation detected with anti-α-Gal IgM (figure 11B). Higher molecular weight proteins as well as proteins of molecular weight similar to AP-N (approx. 116 kDa) were detected in B11 and C9. Since there was no detection with anti-APN antibody, the mutant proteins were subjected to further characterization as we suspected either AP-N aggregate formation with epitopes no longer detectable by anti-AP-N antibodies or co-elution of cellular glycosylated proteins.

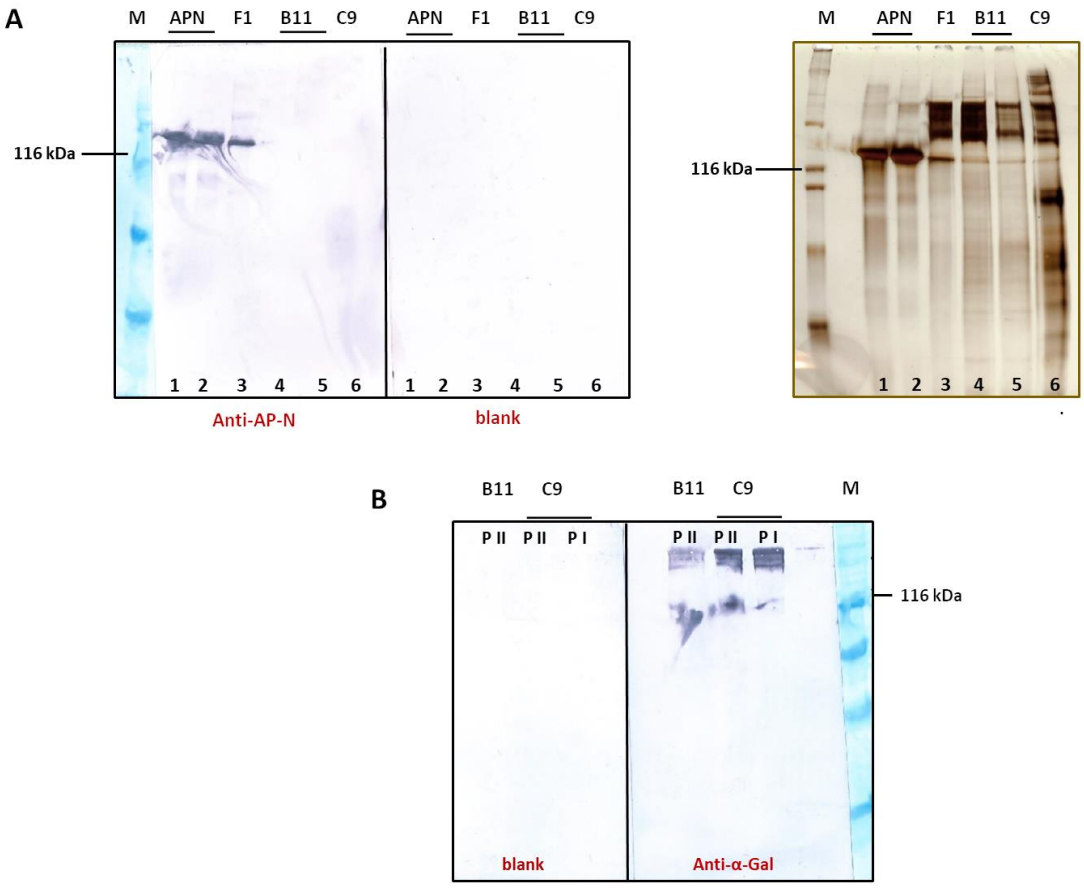


Figure 11. **Western blot analysis of the AP-N mutants**: A. Detection of AP-N protein purified from clones F1, B11, and C9 using anti-AP-N antibody, left. Silver stain gel of pooled HisTrap fractions (positive for AP-N in ELISA) of clones F1, B11, and C9, right; AP-N in lane 1 and 2 is purified non-mutated AP-N protein produced in HEK 293-GT cells. B. Detection of α-Gal expression on AP-N protein from clones B11 and C9 using anti-α-Gal IgM (M86).

4.6 Aminopeptidase N mutant characterization

I further analyzed the mutant proteins to rule out structural changes and to confirm protein identity. Since, detection in ELISA positively shows protein expression but immunoblot with anti-AP-N (clones B11 and C9) were negative for AP-N, further characterization was required. Additionally, since we could confirm presence of α-Gal glycosylation and high molecular weight protein aggregates in silver stain gel we wanted to rule out a possibility of aggregate formation or presence of non-specific protein with α-Gal glycosylation.

Enzymatic activity

Aminopeptidase N is a metalloprotease which exists in two conformations – close and open; it exhibits catalytic activity in its closed confirmation. The enzymatic activity can be checked using a substrate L-leucine-p-nitroanilide. AP-N in its native form catalyzes conversion of L-leucine-p-nitroanilide to p-nitroanilide which results in fluorescence. By measuring the fluorescence of the protein its structural integrity can be confirmed (Chen et al. 2012). Therefore, enzymatic assay was perfomed on mutant as well as native AP-N preparations. Figure 12 shows catalytic activity of the mutant AP-N protein as well as native (APN_C) and recombinant AP-N (APN+ and APN-) which is based on increase in optical density over time.

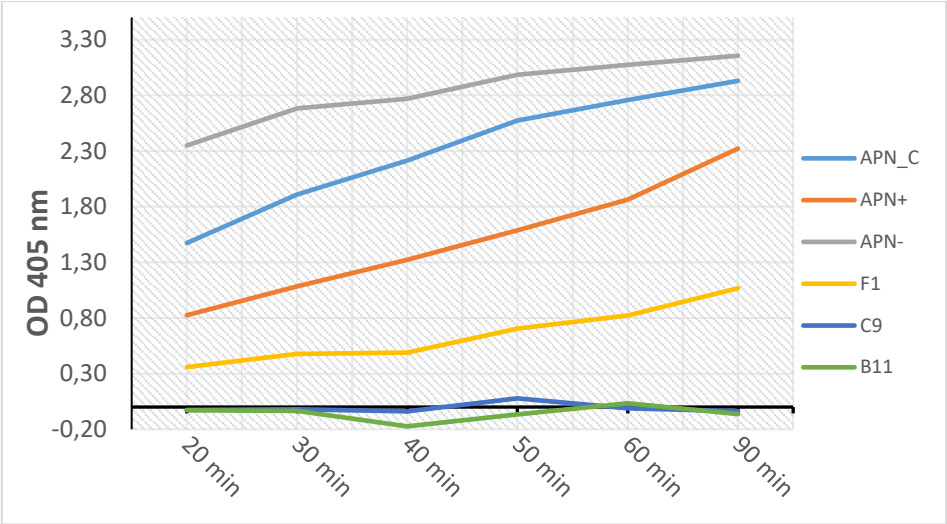


Figure 12. **Enzymatic activity assay showing catalytic activity of AP-N protein and mutants**: Catalytic activity analyzed using L-leucine-p-nitroanilide (10 mM) as substrate. F1, B11, and C9 are the mutant proteins. APN+ (α-Gal glycosylated AP-N), APN_C (commercial porcine AP-N) and APN- (without α-Gal glycosylation) are not mutated. X-axis – enzyme activity time, y-axis – optical density at 405 nm.

Recombinant AP-N expressed in HEK cells without α-1,3-GT activity (APN-) exhibited highest product formation followed by native AP-N (APN_C, commercial AP-N) which is a commercial preparation obtained by extracting AP-N from pork kidney. AP-N with α-1,3-GT activity showed reduced activity (APN+), whereas activity decreased significantly in F1 clone and no activity could be detected in clones B11 and C9. Most likely there is a direct correlation between product formation and AP-N activity, another reason for difference, say, among un-mutated AP-N could be a difference in purity of the protein samples.

Allergenicity determination

Allergenicity of mutant protein was determined in basophil activation test using whole blood from an α-Gal-allergic patient. Briefly, mutants (F1/C9/B11) and controls (positive, aGalAPN/comPN/anti-FcεRI/fMLP; negative, wtAPN) were added to patient whole blood at five concentrations (1 ng/ml – 10 µg/ml) and stained with marker antibodies as per standard BAT protocol [30, 31] followed by detection of %CD63 upregulated cells in flow cytometry.

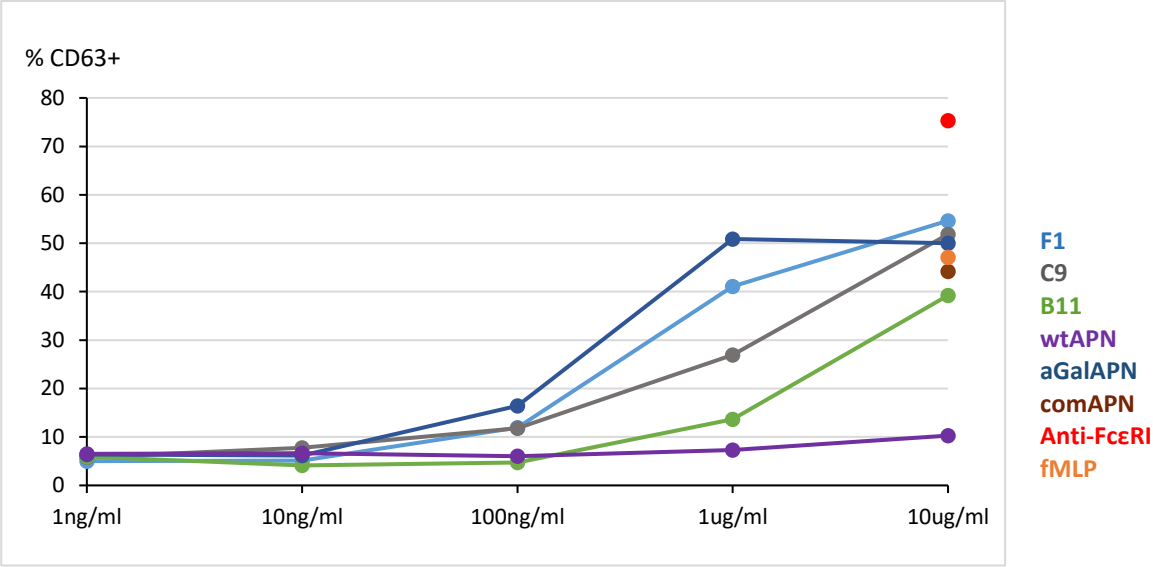
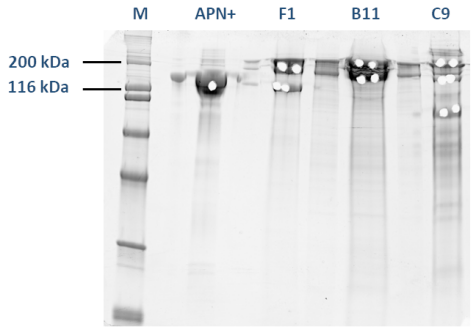


Figure 13. **Mutant AP-N proteins are allergenic when analyzed in basophil activation test (BAT)**: All three mutant proteins (F1, C9, and B11) were analyzed in a BAT assay using whole blood from an α-Gal-allergic patient. aGal APN (α-Gal glycosylated AP-N) and commercial AP-N from porcine kidney (comAPN) serve as positive control and wtAPN (AP-N without α-Gal glycosylation) is the negative control. X-axis: allergen concentration; y-axis: %CD63 positive cells.

Previously, α-Gal glycosylation were detected in all mutants in western blot analysis using anti-α-Gal IgM (M86) and the result is further confirmed in BAT assay. Basophil degranulation (%CD63 positive cells) confirms presence of α-Gal in these protein fractions. The activity could be directly correlated to number of α-Gal epitopes present, aGalAPN (AP-N with α-Gal) shows highest sensitivity followed by F1, whereas C9 and B11 show reduced reactivity in comparison. AP-N as expected didn't induce basophil reactivity due to the absence of α-Gal epitopes.

Proteomics analysis

Enzymatic assay and basophil activation test led us to two conclusions – firstly, no enzymatic activity can be seen in B11 and C9, which would point towards either loss of structural integrity upon mutation, or perhaps there's no AP-N present in the purified fractions. Secondly, a positive basophil response was observed which confirms presence of α-Gal in the protein fractions. Therefore there exists two possibilities – either changes in AP-N structure leading to a loss of its enzymatic activity or there is no AP-N production and fractions inducing basophil reactions are non-specific human proteins that exhibit α-Gal glycosylation due to α-1,3-GT activity in the genetically modified HEK 293 cells. To confirm this, 5 µg of each mutant preparation were loaded onto precast MiniProtean (AnyKD^{TM}, Biorad) gel and resolved by electrophoresis followed by staining with Sypro-Ruby to visualize protein fractions, fractions close to expected band size were excised (figure 14) and subjected to proteomics analysis. Table below (figure 14) shows identified proteins from each fraction.



Lane	Identified proteins	Gene name
APN	sp|P15145|AMPN_PIG, A0A0A0MRA3, Q7Z5L0, A0A0A0MRP6	AMPN_PIG, VMO1, TTN, SMARCA1;SMARCA5
B11_1	P24821, M0R2U2, Q7Z5L0	TNC, FBL, VMO1
B11_2	P01023, K7EIN7,C9JXB8	A2M, MKNK2, RPL24
C9_1	P01023, P24821, A0A3B3IRY2	A2M, TNC, AP5Z1
C9_2	P01023, P24821, Q7Z5L0, A0A0A0MRA3	A2M, TNC, VMO1, TTN
C9_3	P01023, P24821, A0A087WYL7, D6RHW0, C9JXB8	A2M, TNC, MED6, SLC38A9, ATP6AP1L
F1_1	sp|P15145|AMPN_PIG, P24821, Q7Z5L0, Q9UHG3	AMPN_PIG, TNC, VMO1, PCYOX1
F1_2	P24821, A0A0A0MSP7	TNC, FRMPD3

Figure 14. **Proteomics analysis to detect AP-N in purified mutant protein**: A. Sypro-Ruby stained gel showing bands picked for the analysis. APN+ (α-Gal glycosylated AP-N), F1 (50% sites mutated), B11 (glycosylation at distant positions – 3 sites), and C9 (glycosylation near-by – 3 sites). B. Proteomics analysis shows detection of several human proteins in all samples; porcine AP-N (AMPN_PIG) is detected in APN+ and F1 (fraction F1_1) samples. Marker (M) – broad range (Biorad).

Porcine AP-N (AMPN_PIG) is successfully detected in APN+ (AP-N with α-Gal) and F1 (F1_1) fractions whereas no AP-N could be detected in any of the fractions from B11 and C9 clones. Thereby confirming no expression of mutant AP-N and thus the positive BAT results are due to the presence of α-Gal glycosylation on human proteins which were co-eluted during purification steps. A2M (alpha-2-macroglobulin) contains eight and TNC (tenascin) contains three glycosylation sites.

DISCUSSION

Carbohydrate epitopes present a unique challenge in studying effector cell mechanism due to their variable structural arrangement compared to the widely studied protein allergens. The recent discovery of α-Gal allergy creates a need to better understand the glycan epitope-IgE interaction. α-Gal is a disaccharide structure, galactose-α-1,3-galactose, which is produced by all mammals except humans and old world monkeys due to loss of activity of the enzyme α-1,3-galactosyltransferase [13, 32]. Specific IgE against the α-Gal epitope is produced in individuals upon sensitization by tick bites [33]. Due to requirement of multiple epitopes for cross-linking of IgE to induce effector cell function, glycan-bearing allergen molecules should possess at least two epitopes at optimal distance to be able to cross-link IgE molecules. α-Gal is expressed on both glycoproteins and glycolipids [9]. For glycolipids, formation of chylomicrons is proposed to present multiple α-Gal epitopes [15] which can result in mast cell/basophil degranulation, and as already depicted in chapter III, glycolipid micelles are able to induce degranulation of basophils *in vitro* [16]. Since glycan structures can vary in length as well as in orientation when present on the surface of a protein molecule it's difficult to predict factors contributing to an effective cross-linking. Moreover, the number of glycan epitopes and their proximity on protein surface will perhaps guide successful effector cell degranulation [19]. To this effect, I have made an attempt to produce mutant aminopeptidase N molecules with α-Gal-glycan present at specified positions with a plan to use them in a prospective study to analyze structural characteristics involved in the glycan-IgE interaction. Additionally, we planned to use them in the ongoing sensitization experiment in knockout mouse models (Technical University Münich) and in basophil activation test to evaluate the allergenic potency of these molecules.

11 sites out of the 14 potential N glycosylation sites were mutated from asparagine to alanine. Although mutations were successfully introduced and initial screening of AP-N expression in HEK cells was successful, the clones were difficult to screen and most of them were not stable. When I made an attempt to create a stable cell-line, most clones died and it was only after a few attempts that I could establish few cell-lines of the mutant AP-N clones. This was however only a problem with the final three mutants with 11 mutations (MV I, MV II, MV82), intermediate clones with six mutations were easy to screen. I could isolate several clones and obtain a stable cell-line expressing the mutant AP-N protein, this protein was successfully identified in ELISA, immunoblot and characterized in BAT as well as the enzymatic assay. We suspect that there was a progressive decline in AP-N stability with introduction of more mutations, making it much more difficult to screen. Final mutants with only three glycosylation sites were mostly not expressing any proteins and after many rounds of selection I could establish only a few clones for two mutants out of the three. I stopped selection of the third mutant (adjacent sites, MV82) as I couldn't recruit any clone for it.

Next, I detected the expressed mutant proteins for C9 and B11 in ELISA screening after purification, however, these mutant proteins were not recognized by anti-APN IgG in a western blot whereas high-molecular weight bands were detected using anti-α-Gal antibody (M86). This gave rise to two possibilities either mutations led to a heavily distorted protein structure that resulted in a loss of anti-APN antibody binding site or unspecific cellular proteins with α-Gal epitopes were detected in the purified fraction. Therefore, in the next step I characterized these molecules in basophil activation test as well as in enzymatic assay to detect allergenicity of purified proteins and to analyze the structural integrity of the mutant AP-N. In line with the western blot results, BAT assay using blood from α-Gal-allergic patient exhibited basophil degranulation with all mutants showing presence of α-Gal epitope, however, as mutants were expressed in HEK cells with α-1,3-GT activity it is expected that α-Gal can be expressed on non-specific human proteins which are co-eluted in the chromatographic purification.

Enzymatic assay used to detect activity of AP-N molecule was not successful pointing towards either major structural change that can make the catalytic site inaccessible or no production of the AP-N molecule at all. Therefore, finally we decided to perform proteomics analysis to detect mutants as well as any unspecific protein that might have been co-eluted. We couldn't detect any AP-N mutant protein in fractions from clones C9 and B11.

Since we could successfully detect F1 (intermediate) protein that had 6 mutations, it is highly likely that proteins became very unstable upon further mutations and perhaps introduction of 11 mutations was severely destabilizing the AP-N molecule. The fact that the production yield of F1 protein was much lower than that of the original AP-N supports this assumption. In an attempt to understand the effect of alanine substitution on AP-N stability, I have analyzed the amino acid substitutions using an online tool, Dynamut (data not shown). Change in Gibbs free energy is measured to predict stabilizing/un-stabilizing effect of substitution. In this model, six changes of substitution (from asparagine to alanine) were destabilizing and therefore different amino acids should be used for substitution. A preliminary analysis showed increased stability when substituting asparagine with glutamic acid due to structural similarity, however not all 14 positions showed a stabilizing effect. Hence another option could be introducing a combination amino acid replacements by analyzing different amino acid substitutions and choosing most stabilizing exchange for each position. Interestingly, when introducing the original amino acid sequence of AP-N in 'Robetta', the structure looked very similar to the mutant structures and did not match the crystal structure of AP-N (PDB 4FKK) which was produced in insect cells and carried carbohydrates. This could point to the fact that carbohydrates might be crucial for AP-N stability, explaining our inability to express AP-N devoid of 11 carbohydrates. However, further modelling and stability analysis was beyond the scope of this project but could be further pursued. Due to time constraints, we decided to not further analyze the reason for the lack of detectable protein expression. Misfolded mutated protein could be either rapidly degraded upon translation or be unstable after secretion due to the lack of carbohydrates. Another important aspect which should be checked is the formation of protein aggregates which could be the case in the presence of misfolded proteins.

In this project, we had chosen porcine AP-N as model protein because it has been characterized as major α-Gal carrying protein in pork kidney, a potent trigger of anaphylaxis for α-Gal allergic patients. The structure has been resolved and the high number of glycosylation sites made it an attractive model for the design of mutants carrying α-Gal at different locations in order to explore the spatial constraints of IgE-binding and effector cell activation. Unfortunately, that approach failed and further research is needed in order to design carrier molecules that would be stable enough to support either the elimination or the addition of glycosylation sites in a defined spatial arrangement. Furthermore, another bottleneck of such an approach is certainly the lack of controlled expression of α-Gal glycosylation. Although α-Gal will be present at the terminal end of the majority of carbohydrates, the structure of those carbohydrates can be very complex and α-Gal can be present on mono-, bi- or tri-antennary complexes as shown in chapter III. This process depends on many factors and cannot be controlled. An alternative method could be the chemical engineering of α-Gal epitopes to a carrier protein at defined sites. The availability of well-defined synthetic α-Gal carriers would be an asset to study mechanisms of immune responses to carbohydrates in allergen sensitization, but also during the effector phase.

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DISCUSSION AND PERSPECTIVE

This thesis was embedded in a large collaborative effort with two major objectives: the investigation of sensitizing mechanisms induced by tick bites and the characterization of the molecules implicated in delayed allergic reactions to red meat. Chapter I analyses human humoral immune responses to tick bites. For this part of the project, we took advantage of serum samples and questionnaires previously collected from forestry workers from Luxembourg in the context of a Lyme disease study. Chapter II investigates the tick as sensitizing source. Ticks were fed and kept under controlled conditions for defined time intervals by our collaborators at the University of Hohenheim and transferred to LIH for proteomics analysis. Chapter III studies the allergenicity of glycoproteins and glycolipids of mammalian meat and their role in delayed anaphylaxis. Preliminary work in the group identified pork kidney as a potent trigger of allergic reactions [1] and further identified two metallopeptidases as α-Gal carrying molecules in pork kidney [2]. Following these studies, we adopted pork kidney as a model food source to study the mechanisms behind delayed onset of reaction in meat allergic patients. Additionally we have used the identified molecule (Aminopeptidase N) to create mutant proteins, which we aimed at using to study the role of spatial distribution of α-Gal glycans on effector functions. In this thesis, I have built on the existing knowledge to answer some of the questions on mechanistic and molecular aspects of the red meat allergy.

Chapter I: Anti-α-Gal IgG/IgE profiling in a high-risk cohort of Luxembourgish forestry employees

Chapter I presented here aimed at investigating IgG and IgE antibody responses in an occupational population exposed to repeated tick bites. Although IgG responses have already been characterized in meat-allergic patients and in healthy controls [3-6], there is a lack of data related to individuals exposed to ticks. The study provides a detailed characterization of a high-risk cohort of forestry employees from Luxembourg. Through a collaboration with Prof. C Muller, LIH, we were able to retrospectively analyse questionnaires and serum samples obtained in 2012/2013 from 219 participants who were employed as workers, drivers, and rangers in the forestry service department of Luxembourg. Since red meat allergy is a tick borne disease, individuals more exposed to tick bites are naturally at a greater risk of being sensitized and eventually developing allergy to mammalian meat. Sera from forestry employees were first probed for presence of specific IgE against the disaccharide α-Gal in ImmunoCAP, then levels of total IgE and IgG subclasses against α-Gal were measured in ELISA. We found a high prevalence rate of sensitization (21%) among employees, which is in line with previous reports, confirming a similar observation in Luxembourg as previously reported in Germany and Spain [7, 8]. This first national study for Luxembourg clearly shows a need for preventive measures in individuals at a high risk of tick exposure, not only to prevent pathogen transmission, but also to prevent red meat allergy. We additionally investigated if demographic data were linked to sensitization, but found no significant difference in sensitized *versus* non-sensitized individuals in terms of age, hours spent outside, years in occupation or number of tick bites experienced. The only highly significant difference was that individuals with an atopic background were more likely to have sIgE to α-Gal than non-atopic individuals (OR of 5.7), pointing to the fact that an atopic background also favors Th2 responses to tick bites.

Both sensitized and non-sensitized forestry employees exhibited high levels of α-Gal specific IgG that most likely can be attributed to tick bites as lower levels were observed in a group of individuals reporting a non-related food allergy and in a small group of individuals recalling no history of tick bites. When comparing anti-α-Gal IgG subclasses between sensitized/non-sensitized forestry employees and individuals with a non-related food allergy, the most striking features were elevated IgG1 and IgG2 levels in forestry employees, compared to food-allergic patients. Although food-allergic patients, in our case fish-allergic patients, show a low IgG2 response, IgG1 are completely absent. This finding is in line with data on blood donors, confirming an IgG2 response to α-Gal in controls. A recent study

investigating a concentrated pharmaceutical human IgG preparation found that 82% of the anti-α-Gal antibodies were of the IgG2 subclass, only 14% were IgG1, although the overall subclass distribution is 60% IgG1 and 32% IgG2, showing thus a clear preference for IgG2 in a normal population [9, 10]. Whereas IgG2 antibodies against α-Gal are thought to be induced by the intestinal microbiome, it is tempting to speculate that the break of barrier by the tick bite preferentially induces IgG1 responses to α-Gal while boosting pre-existing IgG2 responses. In individuals with an atopic background, the Th2 environment induced by tick saliva would subsequently promote more easily a direct switch from IgG1 to IgE antibodies. Previous studies have reported increased IgG1 and IgG2 against α-Gal in meat-allergic patients [5, 6], however our study is the first to observe significantly higher levels in a well characterized tick-exposed cohort.

When comparing IgG levels to α-Gal in meat allergic patients to IgG levels to fish allergens in a group of individuals with fish allergy, we observe two interesting patterns: i) IgG levels to α-Gal are much higher than IgG levels to fish allergens, ii) α-Gal allergic patients show almost no detectable IgG4 response to α-Gal whereas fish-allergic patients have highly significant levels of IgG4 to fish allergens. This difference in IgG subclasses in response in a carbohydrate- versus a protein-mediated allergy may point towards significant differences in immune cell (T and B cells) activation which should be further explored. An interesting finding is the lack of a IgG4 response in α-Gal allergy, IgG4 is a hallmark of tolerance development and is reported in patients that undergo immunotherapy or exhibit natural resolution of allergy over time [11].

A limitation of our study is the lack of data on clinical symptoms related to red meat consumption in forestry employees. As this was a retrospective study, we were however not able to retrieve such data. Another limitation is the lack of a normal population to study the sensitization prevalence rate in Luxembourg and the IgG subclass responses in the general population

In summary, we provided evidence for a continuous stimulation of α-Gal related immune responses by repeated bites, translating into elevated levels of IgG1 and IgG2 antibodies directed against α-Gal. This is also reflected by a high prevalence of IgE-sensitization that is much more prominent in the sub-group with an atopic background.

Chapter II: Retention of host blood protein in Ixodes ricinus after moulting and upon prolonged starvation

The correlation between red meat allergy and tick bites is well established and several studies have been published that show presence of α-Gal-carrying molecules in tick saliva, salivary gland, and midgut [12-16]. However, the origin of these molecules within ticks remains a matter of debate as ticks naturally lack α-galactosyltransferase activity and therefore cannot produce α-Gal glycosylation on proteins and lipids. As α-Gal is detected in unfed ticks [14], and bites by unfed nymphs/adults lead to sensitization in an α-1,3-GalT KO mouse model [17], endogenous α-Gal production is hypothesized to play a role in sensitization to α-Gal. Although a role of β4-GALT and α4-GALT genes in α-Gal synthesis was identified [18], another study by Sharma et al. report no reduction in the level of detected α-Gal after silencing β-1,4-GALT. However, they do report significant reduction upon silencing α-D-Galactosidase which is important for galactose metabolism and is proposed to be used by ticks to remove α-Gal from host proteins and lipids [19]. The term 'unfed ticks' refers to ticks that have been fed at a previous developmental stage and that have been allowed to moult before being used in the experiment. Ticks are blood-sucking parasites and they can survive for several months without feeding.

It is therefore tempting to speculate that α-Gal carrying mammalian blood components could be stored in specific tick compartments and could be injected into human hosts upon a tick bite. The fact that α-Gal has been detected in tick salivary gland and gut further supports this assumption.

In **chapter II**, we have analysed the presence of host blood protein in *Ixodes ricinus* ticks after moulting and starvation. The objective of our longitudinal experimental setup was to investigate the presence of mammalian proteins in so-called unfed ticks and to demonstrate that mammalian protein can be detected after a prolonged time of starvation. We fed *Ixodes ricinus* at larval stage and allowed them to moult and hence analysed unfed nymphs immediately after moulting and again at two time-points after periods of prolonged starvation.

Most sensitization experiments reported to date were performed using either unfed nymphs or adults giving an impression of a role of endogenously produced α-Gal in induction of specific IgE. However, in those studies and in studies reporting detection of α-Gal-carrying proteins in ticks, the origin of the molecule (tick/host) is not clear. Therefore, although endogenous expression of α-Gal in ticks has been proposed we second a role of retained host blood glycoproteins or glycolipids as a sensitizing source. In the current study, we have identified various mouse-originating proteins that were detectable in nymphs even after starvation of five months. Another important observation came from analysing the iBAQ intensity, which is a proteomic analysis method giving an insight into the relative abundance of proteins. Upon comparison of mouse peptides and tick peptides detected in all three longitudinal samples (0 weeks, 10 weeks, and 20 weeks) we found similar quantities of both mouse and tick proteins, which is a highly interesting information as only approximately 60 peptides were detected of mouse origin compared to thousands of tick peptides. This shows the presence of relatively high quantities of mouse protein in unfed nymphs even after a long starvation. Due to the low quantity of biological material, a glycomic analysis of the identified mouse proteins was not possible. We therefore were not able to provide a direct confirmation that retained host protein is a source of α-Gal. Although this is certainly a limitation of the study, we identified for the first time mouse proteins in *Ixodes ricinus* nymphs, a prevalent vector of α-Gal sensitization in Europe. It is to be expected that the identity and number of retained host proteins as well as their glycosylation status is very much dependent on the last host of the tick and the time span between feeding and next bite. Our study does not exclude that endogenous production of α-Gal plays a role in sensitization to α-Gal, it rather cautions against regarding unfed ticks as free of mammalian material and thereby postulating endogenous α-Gal production as the only source of α-Gal.

In the frame of our collaborative project, Dr. J. Fischer detected α-Gal-carrying molecules in fed and unfed adult female *Ixodes ricinus* ticks. Most importantly, α-Gal was not ubiquitously expressed in tick tissue, but restricted to salivary glands, midgut, and hemolymph [14]. Although the study was not able to distinguish between exogenous and endogenous α-Gal production, these findings suggest that α-Gal epitopes are related to specific metabolic pathways. A next step could be using metabolically labelled host glycoproteins to study the incorporation of host blood and localization in various compartments within ticks. The potential role of glycolipids, which are also abundantly found on rabbit erythrocytes (see chapter III), remains to be elucidated.

Chapter III: Role of glycolipids versus glycoproteins in delayed occurrence of symptoms in α-Gal allergic patients

A key factor behind the late discovery of α-Gal allergy is delayed occurrence of symptoms, often 3-6 hours after food consumption, which made it difficult to correlate symptoms to the food consumed [20]. Moreover, even after a decade of discovery the exact mechanism behind delayed occurrence of symptoms remains somewhat enigmatic. A key distinguishing feature of red meat allergy is an immediate hypersensitivity reaction upon intravenous injection of biologicals and occurrence of a delayed response upon ingestion, thereby pointing towards a delay in allergen availability when an oral route is involved. An *ex vivo* study correlating meat consumption to onset of symptoms successfully showed basophil activation within 3-6 hours after oral introduction of pork sausage [21]. This further led to speculation on the involvement of glycolipids in mediating reactions as they exhibit a slower digestion kinetics and therefore probably contributing to a delayed appearance of allergen in the bloodstream. A recent study further strengthened the finding by showing packaging of α-Gal-bearing glycolipids into chylomicrons using an *in vitro* model of a Caco-2 monolayer [22]. However many questions remained unanswered such as allergenic potential and IgE-reactivity of α-Gal-carrying glycolipids, the structural feature leading to cross-linking of IgE on basophils and mast cells and most importantly how they compare to α-Gal-carrying glycoproteins. Since both proteins and lipids can bear α-Gal, another important aspect is to understand the role of the carrier molecule in imparting the characteristic delayed response.

In **chapter III**, I have explored the allergenic capacity of α-Gal-carrying glycolipids and compared it to α-Gal-glycoproteins. Another question that we have investigated in the context of delayed symptoms is the stability of α-Gal-carrying glycoproteins upon gastric digestion. Previous studies on protein allergens have shown an increased resistance to gastric digestion due to the presence of glycosylation of proteins [23]. A recent study using bovine serum albumin (BSA) carrying a synthetic α-Gal epitope found that glycosylated BSA showed a reduced susceptibility to gastric digestion and hampered transcytosis through a Caco-2 cell monolayer [24]. Studies by our collaborations partners Fischer and Morisset had previously identified a decreased reaction time and increased symptom severity in meat-allergic patients upon consuming pork kidney, possibly due to a higher abundance of α-Gal epitopes in pork kidney [1, 25]. We therefore in this chapter additionally analysed the stability of allergen molecules (glycoproteins) in pork kidney, both raw and cooked, in a simulated *in vitro* gastrointestinal digestion assay.

We isolated α-Gal carrying glycolipids from rabbit erythrocytes and characterized their allergenicity and IgE-reactivity using patient whole blood in basophil activation tests and serum in ELISA and immunoblot analysis. Glycolipids were found to bind IgE and activate basophils, although they were less potent than a commercially available α-Gal-carrying glycoprotein (α-Gal-HSA). Glycolipids formed micelles which allows presentation of multiple α-Gal epitopes, thereby enabling IgE cross-linking on effector cells. The micelle size observed *in vitro* was in the same range as normally observed for chylomicrons in human plasma (100 – 1000 nm) and thus mirrors *in vitro* the simulation of a probable presentation of glycans, when packaged in chylomicrons, to cell surface-bound IgE *in vivo* [26]. Next, we sought to understand the interesting observation of a rapid and more potent allergic response in case of pork kidney (PK) consumption. Patients react to as little as 1-2 g of PK which is most likely due to the high abundance of α-Gal glycoproteins in this food source [1, 2]. PK is widely studied in xenotransplantation research and is known to express α-Gal on both glycoproteins and glycolipids. We therefore explored PK in detail to understand the role of glycoproteins and glycolipids. We quantified α-Gal in the protein and lipid fraction of PK by inhibition ELISA and compared the allergenic potential of both fractions in basophil activation test. In thin layer chromatography, we identified short-chain

glycolipids carrying α-Gal, but due to their low abundance we couldn't identify any α-Gal-carrying glycolipids in glycomic analysis. The high content of α-Gal-carrying glycoproteins and the very low content of α-Gal-carrying lipids helped us to clearly establish a major role for glycoproteins in PK related allergic symptoms. We performed gastro-intestinal digestion assays of raw and cooked PK to measure the stability of allergenic glycoproteins and detected stable α-Gal glycosylated peptides as well as intact AP-N protein after up to 4 hours of digestion in our *in vitro* setup. This longer stability of glycosylated proteins would explain symptom occurrence in 1-2 hours of consumption, as glycoproteins have to be processed into smaller glycopeptides to be absorbed in the gut. Another supporting evidence comes from the study by Eller et al. where they show very clearly a more rapid appearance of α-Gal carrying molecules in blood upon consuming a PK smoothie, facilitating a rapid gastric transition and thus an earlier availability to intestinal digestion and absorption into the blood [27].

After coming to conclusion that both glycolipids and glycoproteins exhibit allergenic potential, we next looked into natural food sources, beef meat and pork kidney, to compare the relative abundance in the respective allergen sources. We found a correlation between the quantities of glycoproteins and glycolipids detected in beef and pork kidney in inhibition ELISA and the EC50 values in the basophil activation test, thereby showing a direct link between relative allergen quantity and basophil activation. We therefore propose that relative abundance and stability of α-Gal-carrying proteins/lipids play an important role in delayed response time as well as the severity of experienced reactions. We observe less reactivity and lower allergen quantity in beef meat compared to PK, which is in line with the clinical observation that muscle meat elicits symptoms less consistently than PK [25]. A second important finding is that glycolipids isolated from food can bind IgE and activate basophils. Thus, our findings suggest that the relative abundance and stability of α-Gal molecules in the food consumed are determining the reaction time and severity in patients.

CONCLUSIONS AND PERSPECTIVES

Red meat allergy or α-Gal syndrome is an enigmatic allergic disease that has several unique characteristics. These are: clinical relevance of IgE antibodies directed against a carbohydrate, sensitization by tick bites leading to food allergy and a delayed occurrence of symptoms upon ingestion of mammalian meat. This PhD thesis succeeded in bringing great novelty to several aspects of the α-Gal syndrome, thereby substantially advancing our current knowledge.

The findings on the retention of mammalian proteins during tick moulting and starvation are crucial to the interpretation of ongoing studies on sensitization to α-Gal in α-1,3-GalT knockout mouse models. Further studies need to establish if other ticks are also able to store host protein over time. Different ticks have different types of life cycles that will also influence host protein storage. Finally, metabolic labelling of host glycoproteins is needed in order to trace the fate of host glycoproteins. The events occurring at the site of the tick bite will also need further investigation by using human biopsies and α-1,3-GalT knockout mouse models.

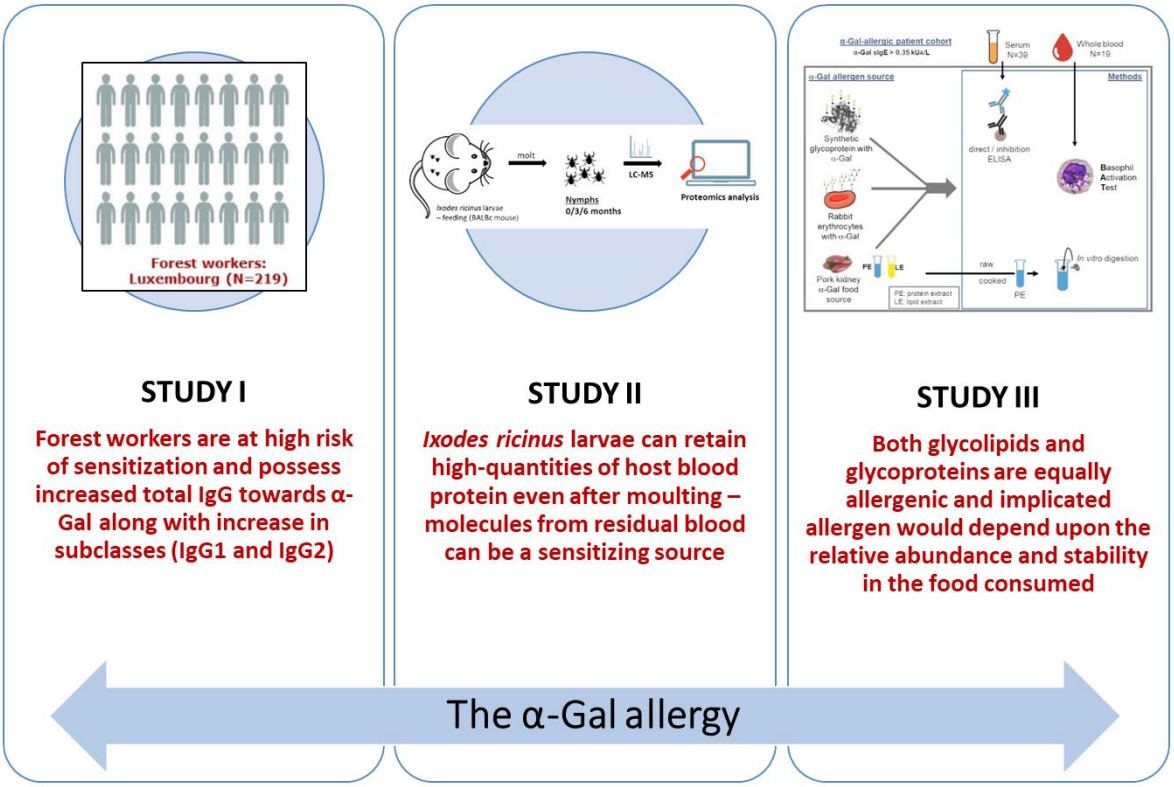
The present thesis was embedded in a large collaborative project that also investigated α-Gal sensitization in α-1,3-GalT knockout mice by intradermal injection of several types of α-Gal carrying molecules. Glycoproteins and glycolipids produced in the present thesis have been used in sensitization and anaphylaxis experiments by the team of Prof. Biedermann, Technical University of Munich. The team could successfully sensitize mice by intradermal injection. Analysis of humoral and cellular responses is in progress and will be the subject of another manuscript. Further studies will be needed to understand the primary IgG2 response to α-Gal, which is thought to be triggered by the intestinal microbiome, and how tick bites can break tolerance in the gut and lead to food allergy.

Another arm of the project, which is complementary to the epidemiological study of the present thesis, is a longitudinal analysis on immune responses to tick bites. Participants with and without sIgE to α-Gal were recruited by Dr. Fischer at the University of Tuebingen, and whole blood samples obtained after tick bites were immunophenotyped at LIH. These analyses will enable identifying the immune cells involved in responses to tick bites. Immune responses towards carbohydrates are largely believed to be T-cell independent but a role of T-cells can be expected when a glycan is presented in conjugation with a protein.

Presently, patients with α-Gal syndrome are advised to avoid mammalian meat. Based on our findings, a characterization of commonly consumed meat and dairy products will allow determining the overall abundance of α-Gal molecules as well as the relative abundance of glycoproteins or glycolipids and their allergenic potential. Their allergenicity will depend upon the number of α-Gal-carrying epitopes present and the stability of the molecule. This can serve as a reference guide for dietary recommendation to patients. On the other hand, the identification of α-Gal-carrying glycolipids as IgE-binding molecules will enable further studies on allergen presentation by antigen presenting cells, absorption through the intestinal barrier, and activation of effector cells.

Summary of key findings

In conclusion, the work done in this thesis has substantially contributed at increasing our understanding of α-Gal allergy by exploring three different, but interconnected domains: immune response of individuals exposed to recurrent tick bites, proteomic analysis of the tick, a dominant sensitizing source and molecular insight into the delayed onset of the allergic response. Most important findings being – role of tick bites in induction of specific IgG to α-Gal not only in patients and sensitized individuals but as well in non-sensitized individuals; secondly we report retention of high quantities of host blood in *Ixodes ricinus* challenging any final conclusion on the origin of sensitizing molecules in ticks. Lastly, we show a direct role of α-Gal in the allergic response irrespective of the carrier molecule. Below is a summary of key findings from these three studies:



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ANNEXURE

ANNEXE I

AWARDS AND RECOGNITION

I. Nachwuchsförderpreis (Young Talent Award) 2022 – Deutsche Gesellschaft für Allergologie und Klinische Immunologie (DGAKI)
For the published article: "α-Gal present on both glycolipids and glycoproteins contributes to immune response in meat-allergic patients." *The Journal of Allergy and Clinical Immunology*

II. Congress Grant for a short oral presentation – European Academy of Allergy and Clinical Immunology (EAACI) Annual Conference, Prague 2022
Topic: "Mouse blood protein survives degradation in *Ixodes ricinus* ticks after moulting and upon starvation up to six months"

III. Oral presentation – Food Allergy and Anaphylaxis Meeting (FAAM), online 2021
Topic: "Role of glycolipids in IgE-responses towards α-Gal in patients with red meat allergy"

IV. Best poster presentation – Allergy School EAACI, Paris 2019
Poster presentation: "α-Gal carrying glycolipids show *in vitro* allergenicity in patients with red meat allergy"

V. Travel grant – International Symposium on Molecular Allergology (ISMA), Amsterdam 2019
Poster presentation: "α-Gal carrying glycolipids show *in vitro* allergenicity in patients with red meat allergy"

ANNEXE II

LIST OF PRIMERS

I. LIST OF AMINOPEPTIDASE N MUTAGENESIS PRIMERS

AA position	Sequence (5' --> 3')	Length	Melting temp. (T_m)
Asn 82	5'-gttgcctgattcctacgccgtgacgctgagaccc-3'	34	78.6°C
Asn 124	5'-ccatagcaagaagctcgcctacaccacccagggg-3'	34	78.68°C
Asn 229	5'-ccatgaaggccacgttcgccatcactctcatccacc-3'	36	78.83°C
Asn 237	5'-ctcatccaccctaacgcctcacggccctgtc-3'	32	78.50°C
Asn 258	5'-cacttgcagaagaccccgcctggtctgtcactgagt-3'	36	78.83°C
Asn 286	5'-gcgagttccagagcgtggctgaaacggcccaaaatg-3'	36	78.83°C
Asn 314	5'-atggcatgtatgccctggctgtgacaggtcccatcc-3'	36	78.83°C
Asn 328	5'-catcctaaacttctttgccaatcattatgctacatcctacccactcc-3'	47	78.59°C
Asn 506	5'-atgcctttgcctatcaggccaccacctacctggacc-3'	36	78.83°C
Asn 556	5'-cgtggacaccaagacaggagccatctcacagaagcacttc-3'	40	80.12°C
Asn 569	5'-ctcgactccgaatccgccgtcacccgctcctc-3	32	79.78°C
Asn 622	5'-gggtcttgctgaacgtcgccgtgacaggctatttcc-3'	36	78.83°C
Asn 646	5'-gatgattcagcatcagctgcagacagcctgtcggtcatc-3'	40	80.12°C
Asn 736	5'-tccaacatttcgaaactctcactaaagcctggaccgagcgcc-3'	42	80.19°C

II. LIST OF SEQUENCING PRIMERS

Primer name	Target Sequence	Length	T_m
EF-1a Fwd priming site	5'-TCAAGCCTCAGACAGTGGTTC-3'	21	57°C
APN 1_rev	5'-GCAAGTTCCCCCTGGAATTCAC-3'	22	60°C
APN 2_fwd	5'-ACATGTACGAGATGGAGAGTG-3'	21	56°C
APN 2_rev	5'-TGAAGTCGGGCAAGGCAATC-3'	20	59°C
APN 3_fwd	5'-CAAATCCGACCAGATTGCC-3'	19	56°C
APN 3_rev	5'-TCTGAGCATCCACAGCCTTC-3'	20	58°C
APN 4_fwd	5'-ATGCTCTCCAACTTCCTGAC-3'	20	56°C
APN 4_rev	5'-GTTGAAGCTGTCGTAGATGACC-3'	22	57°C
APN 5_fwd	5'-GTCATCCCTGTCATCAATCG-3'	20	54°C
APN 5_rev	5'-TACCTGTTCAGGAGCCAGAC-3'	20	57°C
APN 6_fwd	5'-GGTAAATGAGGCCGACAAAC-3'	20	56°C
BGH Reverse priming site	5'-TAGAAGGCACAGTCGAGG-3'	18	54°C

ANNEXE III

"A novel monoclonal IgG1 antibody specific for Galactose-alpha-1,3-galactose questions alpha-Gal epitope expression by bacteria." *Frontiers in Immunology* 13:958952

"Luisa Kreft, Aloys Schepers, Miriam Hils, Kyra Swiontek, Andrew Flatley, Robert Janowski, Mohammadali Khan Mirzaei, Michael Dittmar, **Neera Chakrapani**, Mahesh S. Desai, Stefanie Eyerich, Li Deng, Dierk Niessing, Konrad Fischer, Regina Feederle, Simon Blank, Carsten B. Schmidt-Weber, Christiane Hilger, Tilo Biedermann and Caspar Ohnmacht"

DOI: https://doi.org/10.3389/fimmu.2022.958952

Personal contribution: In this project, I have contributed by providing glycolipids which form the part of data represented in figure 1 and 4. I have also participated in the final revisions of the manuscript.

frontiers | Frontiers in Immunology

TYPE Original Research
PUBLISHED 05 August 2022
DOI 10.3389/fimmu.2022.958952

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Microbial Immunology, a section of the journal Frontiers in Immunology

RECEIVED 01 June 2022
ACCEPTED 18 July 2022
PUBLISHED 05 August 2022

CITATION
Kreft L, Schepers A, Hils M, Swiontek K, Flatley A, Janowski R, Mirzaei MK, Dittmar M, Chakrapani N, Desai MS, Eyerich S, Deng L, Niessing D, Fischer K, Feederle R, Blank S, Schmidt-Weber CB, Hilger C, Biedermann T and Ohnmacht C (2022) A novel monoclonal IgG1 antibody specific for Galactose-alpha-1,3-galactose questions alpha-Gal epitope expression by bacteria. *Front. Immunol.* 13:958952. doi: 10.3389/fimmu.2022.958952

A novel monoclonal IgG1 antibody specific for Galactose-alpha-1,3-galactose questions alpha-Gal epitope expression by bacteria

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The alpha-Gal epitope (α-Gal) with the determining element galactose-α1,3-galactose can lead to clinically relevant allergic reactions and rejections in xenotransplantation. These immune reactions can develop because humans are devoid of this carbohydrate due to evolutionary loss of the enzyme α1,3-galactosyltransferase (GGTA1). In addition, up to 1% of human IgG antibodies are directed against α-Gal, but the stimulus for the induction of anti-α-Gal antibodies is still unclear. Commensal bacteria have been suggested as a causal factor for this induction as α-Gal binding tools such as lectins were found to stain cultivated bacteria isolated from the intestinal tract. Currently available tools for the detection of the definite α-Gal epitope, however, are cross-reactive, or have limited affinity and, hence, offer restricted possibilities for application. In this study, we describe a novel monoclonal IgG1 antibody (27H8) specific for the α-Gal epitope. The 27H8 antibody was generated by immunization of *Ggta1* knockout mice and displays a high affinity towards

10.3389/fimmu.2022.958952

synthetic and naturally occurring α-Gal in various applications. Using this novel tool, we found that intestinal bacteria reported to be α-Gal positive cannot be stained with 27H8 questioning whether commensal bacteria express the native α-Gal epitope at all.

KEYWORDS

alpha-Gal, α-Gal, IgG, monoclonal antibody, carbohydrate, red meat allergy, xenotransplantation, bacteria

1. Introduction

Carbohydrates are vital and highly diverse structures that are variable between species. Of note, the alpha-Gal (α-Gal) epitope is a carbohydrate immunogen in humans that has relevance in allergy and xenotransplantation. The determining structure of the epitope is the disaccharide galactose-α1,3-galactose (Gal-α1,3-Gal), which naturally occurs as the trisaccharide galactose-α1,3-galactose-β1,4-N-acetylglucosamine (Gal-α1,3-Gal-β1,4-GlcNAc) on glycosylated proteins or lipids (1). The immunogenic property of the α-Gal epitope in humans is based on the loss of the enzyme α-1,3-galactosyltransferase (GGTA1) in Catarrhines, including apes and humans, which catalyzes the reaction of Gal-β1,4-GlcNAc–R + UDP-Gal to Gal-α1,3-Gal-β1,4-GlcNAc-R + UDP (2). Humans therefore do not express the α-Gal epitope in contrast to non-primate mammals. This absence eventually allows for the sensitization of humans and a subsequent development of the so-called 'α-Gal syndrome' or red meat allergy that is based on the formation of IgE molecules against α-Gal *via* tick bites (3–5). These IgE molecules may lead to allergic reactions including fatal anaphylaxis following ingestion of mammalian meat or related products such as gelatin or innards, for instance pork kidney, which are major sources of allergen in α-Gal-induced meat allergy (6–9). Moreover, sensitization to α-Gal can also result in severe allergic reactions in cancer patients who receive Cetuximab, a chimeric human-murine monoclonal antibody that contains α-Gal on the Fab fragment (10). Interestingly, antibodies of different isotypes against the α-Gal epitope are quite abundant in humans with IgG levels estimated to range between 1% (11) to 0.1% of total plasma IgG with high variability between subjects and lowest abundance in individuals carrying the blood type B antigen (12). The latter observation is likely due to the structural similarity between the α-Gal epitope and blood type B antigen, which contains an additional fucose molecule on the second last galactose molecule (1). These human anti-α-Gal antibodies pose a challenge for xenotransplantation, in particular for pig organ transplantation, which was overcome to some extend with developing *GGTA1* knockout (KO) pigs (13–15). The induction of anti-α-Gal antibodies has been hypothesized to be mediated by the gut microbiota, since intestinal bacteria are recognized by anti-α-Gal binding molecules, such as purified polyclonal human anti-α-Gal antibodies (16, 17) or Isolectin B4 from *Bandeiraea simplicifolia* (BSI-B_4) (18–20). Furthermore, antibiotics have shown to reduce preexisting anti-α-Gal antibodies of the IgG isotype (21) and the oral introduction of *Escherichia coli* O86:B7 in *Ggta1* KO mice has been shown to induce anti-α-Gal antibodies (IgG, IgM) (22). BSI-B_4 and another α-Gal-binding lectin from the mushroom *Marasmius oreades* (MOA) (23) have reduced specificity to the α-Gal epitope, as they both also bind to the blood group B antigen. The currently most widely used α-Gal-specific monoclonal antibody is M86, an IgM antibody which was developed by Galili *et al.* in *Ggta1* KO mice (24) and to some degree also chicken single-chain antibody variable-region fragments (scFv) against α-Gal developed by Cunningham *et al.* (25). Neither of the two antibodies has been convincingly shown to stain bacteria to the authors' knowledge. However, the monoclonal antibody M86 was indeed used to show α-Gal expression on parasites such as *Plasmodium* species (18). As the M86 antibody is of the IgM isotype with limited affinity and purification properties, we aimed to establish a novel IgG antibody with high affinity for both the di- and trisaccharide α-Gal epitope and with wide applicability.

Here, we report the development of a novel IgG1 antibody called 27H8 that is highly specific for both synthetic and naturally occurring α-Gal epitopes. The 27H8 monoclonal antibody shows high affinity to the α-Gal epitope and offers wide applicability for α-Gal detection such as in ELISA, dot blots, immunohistochemistry and flow cytometry. Using the 27H8 antibody, we did not find any specific binding to bacteria originating from the intestinal tract while cross-specific BSI-B_4 readily stained cultured or intestinal bacteria. Altogether, our newly developed antibody can be used as a novel tool for α-Gal detection with high sensitivity and specificity. Lastly, our results question the role of the intestinal microbiota as a major source of the α-Gal epitope for sensitization.

2. Material and methods

2.1. Animal ethics statement

Ggta1 KO and wildtype (WT) mice were kept under specific pathogen-free conditions. All interventions were performed in accordance with the European Convention for Animal Care and Use of Laboratory Animals and were approved by the local ethics committee and appropriate government authorities (ROB-55.2-2532.Vet_03-17-68). *GGTA1* KO pigs were developed and maintained according to ROB-55.2-2532.Vet_02-18-56.

2.2. Patient and control sera

Serum samples were retrieved from atopic dermatitis patients (male n=13, female n= 6) with a mean SCORAD of 63 ± 14.2, α-Gal allergic patients with α-Gal-syndrome confirmed by α-Gal specific IgE, medical history or oral provocation test (n=7) or healthy controls (n=17). All individuals gave written consent and the study collection was approved by the local ethics committee. The α-Gal allergic patients and healthy controls were part of the BioBank of the Department of Dermatology and Allergy Biederstein, School of Medicine, Technical University of Munich, Munich, Germany and approved by ethical vote 419/18 S-KK. The atopic dermatitis patient collection was approved by ethical vote 5590/12.

2.3. Immunization protocol and hybridoma generation

α1,3-galactosyltransferase 1 (*Ggta1*) knock-out (KO) mice (26) kindly provided by the group of Florian Kreppel, University of Ulm, Germany were immunized subcutaneously (s.c.) and intraperitoneally (i.p.) with a mixture of 50 µg ovalbumin-coupled Gal-α1,3-Gal-β1,4-GlcNAc trisaccharide (α-Gal-OVA, 14-atom spacer, Dextra, Reading, UK) in 200 µl PBS, 5 nmol CpG2006 (TIB MOLBIOL, Berlin, Germany), and 200 µl Incomplete Freund's adjuvant (Sigma-Aldrich, St. Louis, MO, USA). After 11 weeks, a boost without Freund's adjuvant was given i.p. and s.c. 3 days before hybridoma fusion. Fusion of the myeloma cell line P3X63-Ag8.653 with mouse splenic B cells was performed using polyethylene glycol 1500 according to standard procedure (27). After fusion, hybridoma cells were plated in 96-well plates using RPMI 1640 supplemented with 15% fetal calf serum, 1% glutamine, 1% pyruvate, 1% non-essential amino acids and 2% HAT media supplement (Hybri-Max, Sigma-Aldrich). Hybridoma supernatants were screened 10 days later in a flow cytometry assay (iQue, Intellicyt; Sartorius, Göttingen, Germany) using BSA-coupled Gal-α1,3-Gal (α-Gal-DI-BSA, 3-atom spacer, Dextra) captured on 3D-aldehyde beads (PolyAN, Berlin, Germany). Beads were incubated for 90 minutes (min) with hybridoma supernatant and Atto-488-coupled isotype-specific monoclonal rat anti-mouse IgG secondary antibodies. Antibody binding was analyzed using ForeCyt software (Sartorius). Positive supernatants were further validated by dot blot and cells from clone 27H8 were sub-cloned by five rounds of limiting dilution to obtain stable monoclonal hybridoma cell lines (mouse IgG1/ƙ).

2.4. Purification of the 27H8 antibody

Hybridoma supernatant from subcloned 27H8 was purified on an ÄKTA Pure chromatography system (Cytiva) using Cytiva HiTrap Protein A HP column (Fisher Scientific, Waltham, MA, USA).

2.5. Screening material

Mouse serum albumin (MSA) was purchased from Sigma-Aldrich, Ovalbumin (OVA) EndoFit from *In vivo*Gen, San Diego, CA, USA and Bovine Serum Albumin (BSA) from AppliChem, Darmstadt, Germany (Albumin Fraction V). Further proteins coupled to the α-Gal epitope Gal-α1,3-Gal-β1,4-GlcNAc (referred to as "TRI"-saccharide), Gal-α1,3-Gal-β1,4-GlcNAc-MSA (α-Gal-MSA, 3 atom spacer) and Gal-α1,3-Gal-β1,4-GlcNAc-BSA (α-Gal-TRI-BSA, 3 atom spacer) were purchased from Dextra. α-Gal-rich glycolipids were extracted from rabbit erythrocytes (Innovative Research, Novi, MI, USA) as described previously (28), modified from (29, 30). Bovine thyroglobulin was purchased from Merck, Darmstadt, Germany. His-tagged porcine aminopeptidase N (APN) was recombinantly produced in human embryonic kidney (HEK) 293 cells (APN control without α-Gal) (28) as well as in HEK293 cells stably expressing murine GGTA1 (α-Gal-APN) (31). The cells were cultured in DMEM supplemented with 10% FCS, penicillin and streptomycin. After reaching 70% confluency, FCS-containing medium was removed and cells were gently washed once with PBS. Fresh DMEM (Sigma-Aldrich) containing PeproGrow-1 (serum-free cell culture supplement, PeproTech) was added and cells were cultured for further 4 - 6 days without medium exchange until cell viability showed the first signs of deterioration. Medium supernatant was harvested and passed through a 0.45 µm (Sarstedt, Nürnberg, Germany) filter to remove residual cell debris. The recombinant proteins were purified from the filtrate using Ni-NTA affinity chromatography and subsequent gradient elution with imidazole (AppliChem). Protein-containing fractions were screened for purity *via* SDS-PAGE and subsequent staining with Coomassie blue. Suitable fractions were pooled. Proteins were concentrated using centrifugal filter units (Amicon Ultra-15, Merck), including a final washing step with PBS to reduce the

imidazole concentration to ≤ 20 mM. After sterile filtration (Millex-GV Syringe 0.22 µm Filter Unit, Merck) and shock freezing in liquid N2, proteins were stored at -80°C until usage.

2.6. Screening lysates

Pig wildtype (WT) kidney was derived from a local butcher. Pig KO kidney samples were derived from *GGTA1*-gene knockout (KO) pigs (32). Cultivated *GGTA1* KO and WT pig kidney cells were lysed with Cytobuster (Merck). 0.5 cm x 0.25 cm tissue pieces of pig kidneys (WT/KO) were lysed in 1 ml RIPA buffer containing 50 mM Tris buffer, pH 8.0 (AppliChem), 150 mM sodium chloride (AppliChem), 1% Nonidet P-40 (AppliChem), 0.5% sodium deoxycholate (Sigma-Aldrich) and 0.1% sodium dodecyl sulfate (Sigma-Aldrich). 10 ml RIPA buffer contained 1 tablet Protease Inhibitor Cocktail (Roche, Basel, Switzerland) and 1 tablet PhosSTOP (Roche). Tissue samples were homogenized using metal balls in a TissueLyser LT (Quiagen, Hilden, Germany) at 50 Hz 3 min, sonicated for 10 seconds and centrifuged at 16,000xg for 30 min at 4°C. Protein amounts in the collected supernatants were measured with a Pierce BCA Protein Assay Kit (Thermo Fisher Scientific) using bovine serum albumin (BSA) as standard.

500 µl of whole blood from a donor with blood group B was centrifuged at 2000xg for 10 min and the cell pellet frozen at -80°C before adding 1 ml RIPA buffer containing protease and phosphatase inhibitor as described above. Cells were sheared by massive pipetting and vortexing steps and then incubated on ice for 30 min before centrifuging at 16,000xg for 30 min at 4°C. The supernatant was collected and stored at -80°C until usage.

2.7. Bacterial strains and lysates

Staphylococcus aureus strains Mu50, SA113, COL, 20231, RN1, SH1000, MW2, RN4220, Newman, USA300, *Escherichia coli* strains (K12, DH5α), *Helicobacter pylori* (J99), *Pseudomonas aeruginosa* (DSM 50071), *Haemophilus influenza* (Hi375), *Acinetobacter baumannii* (ATCC 17978), *Salmonella typhimurium* (ATCC 14028) were purchased from ATCC Manassas, VA, USA and DSMZ, Leibniz Institute, Germany. *Akkermansia muciniphila* was obtained from Willem De Vos at Wageningen University. The bacteria were grown overnight at 37°C to a density of 10^9 CFU/ml. All bacteria were grown in Luria Bertani (L.B.) broth (tryptone 10g, NaCl 10g, yeast extract 5g in 1L H_2O, adjust pH to 7.0 with 5 N NaOH, sterilize), except for *H. pylori* in Brain Heart Infusion (BHI) (beef heart, 5 g/L, calf brains, 12.5 g/L, disodium hydrogen phosphate, 2.5 g/L, D (+)-glucose, 2 g/L, peptone, 10 g/L, sodium chloride, 5 g/L) plus 20% fetal calf serum (FCS), *H. influenza* in BHI 37g, NAD 15mg, and Hemine 15mg in 1L H_2O, and *Akkermansia muciniphila* (ATCC BAA-835) in reduced BHI. Pelleted bacteria (approximately $3x10^9$ bacterial cells) were washed with PBS and resuspended in 1 ml RIPA buffer as described for mammalian samples, and added to glass beads and beat for one hour (max speed 2800 rpm using a Vortex shaker) and transferred to new tubes for storage at -80°C.

E. coli HS was originally isolated from a human fecal sample of a healthy adult (33). *E. coli* O86:B7 and *Lactobacillus rhamnosus* were purchased from the American Type Culture Collection (ATCC 12701 and 53103), *E. coli* BL21 from Thermo Fisher Scientific (EC0114). *E. coli* strains were grown overnight at 37°C in LB medium, *L. rhamnosus* was grown overnight at 37°C in Lactobacilli MRS broth (proteose peptone #3 10 g, beef extract 10g, yeast extract 5g, dextrose 20g, sorbitan monooleate 1g, ammonium citrate 2g, sodium acetate 5g, $MnSO_4$ x H_2O 0.05g, Na_2HPO_4 2g in 1L H_2O, adjust pH to 6.5). RIPA buffer was added to cell pellet of 5 ml culture and cells were lysed for 30min at 30Hz with glass beads.

2.8. Enzymatic digestion and cleavage of the α-Gal epitope

Glycolipids were digested by Endoglycoceramidase I (EGCase I) using a ratio of 1 µg Glycolipids per 1 milliunit enzyme in 1x EGCase I Reaction buffer (New England Biolabs, MA, USA) in PBS for 37°C for 16 hours. Precipitated enzyme was removed after heat inactivation for 20 min at 65°C. 2 µg/ml pig kidney tissue lysates were digested with α-Galactosidase from green coffee beans (Sigma Aldrich) at 10 U/ml in 100 mM potassium phosphate buffer, pH 6.5 for 3 hours at room temperature (RT). Ammonium sulfate was removed from α-Galactosidase preparation before digest by pelleting the enzyme through a centrifugation step at 15,000xg for 10 min at 4°C. The supernatant was collected and the pellet resuspended in an equal volume of potassium phosphate buffer. *S. aureus* lysate was digested by adding 5 µl of whole lysate to 5 µl potassium phosphate buffer containing 20 U/ml α-Galactosidase (end concentration 10 U/ml) and further processed as described before. For EGCase I digestion, 10 µl bacterial lysate was digested in 1x EGCase I reaction buffer diluted with PBS and 1 µl EGCase I as described above (end volume 20 µl).

2.9. Dot blot screening approach

Nitrocellulose membranes (Carl Roth, Karlsruhe, Germany) were cut into length of 10 cm x 0.5 cm and 1 µl of sample was applied 1 cm apart to a maximum of 10 samples per membrane strip, except for horseradish peroxidase (HRP) detection for which 2 µl were spotted (Figure 1C). The amount of blotted α-Gal conjugated glycoproteins and proteins devoid of α-Gal was 0.1 µg (Figures 1E, 4A, F, 5B), 1 µg (Figures 1B, D, 2A) or 2 µg

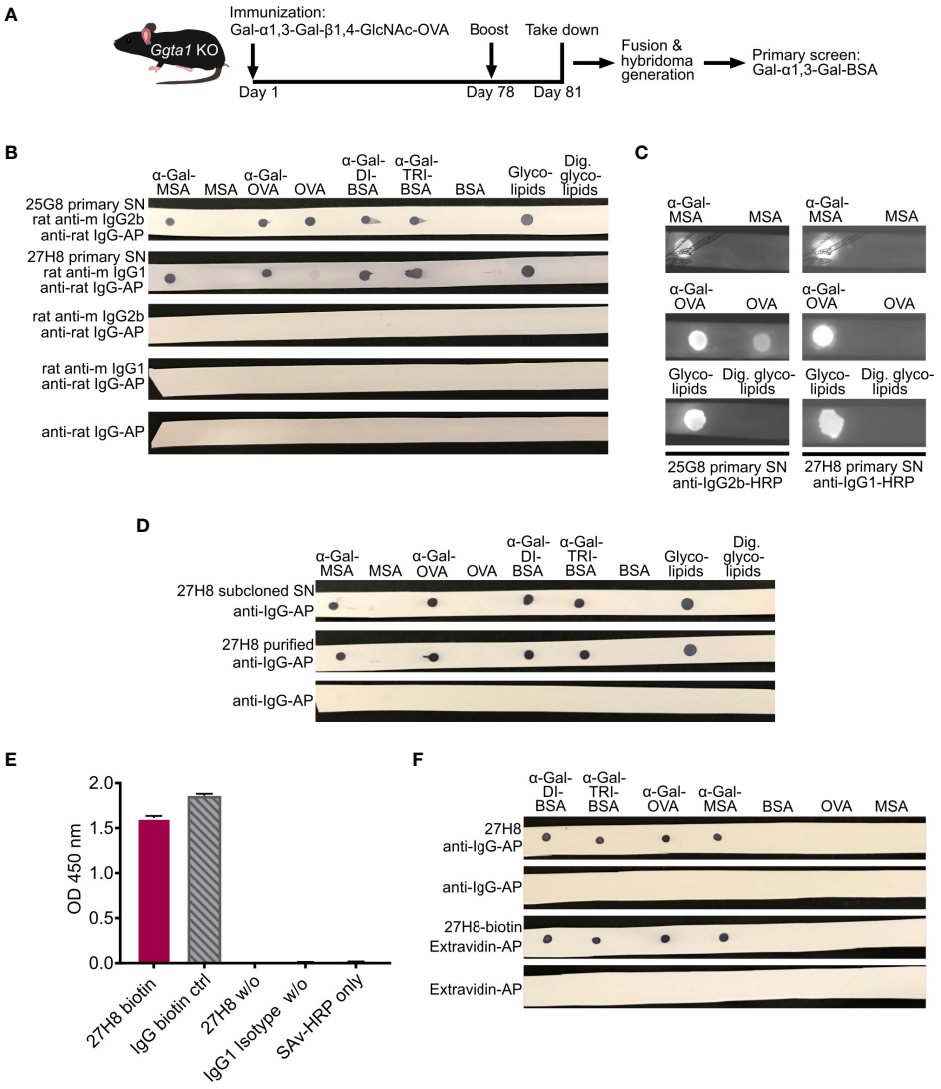

FIGURE 1
Generation, screening and biotinylation of a monoclonal IgG antibody recognizing galactose-α1,3-galactose. **(A)** Schematic approach for the generation of a monoclonal anti-α-Gal antibody through immunization of *Ggta1* KO mice with Gal-α1,3-Gal-β1,4-GlcNAc-OVA (α-Gal-OVA) and screening of primary hybridoma supernatants (SNs) with Gal-α1,3-Gal-BSA (α-Gal-DI-BSA). **(B)** Dot blot of 25G8 and 27H8 primary SNs on α-Gal-conjugated glycoproteins or -lipids and respective negative control proteins devoid of α-Gal. Endoglycoceramidase I (EGCase I)-digested glycolipids (right) served as negative control for glycolipids. Unlabeled rat anti-mouse (anti-m) isotype-specific secondary antibodies and anti-rat-tertiary antibody labeled with alkaline phosphatase (AP) were used for detection. **(C)** Secondary screen on dot blots of 25G8 and 27H8 primary hybridoma SNs on α-Gal carrying glycoproteins and the respective negative control proteins. Detection was performed with HRP-labeled secondary antibodies. See Supplementary Figure 1A for uncropped blots. **(D)** 27H8 subcloned hybridoma SN and 27H8 purified antibody were screened as in **(B)**. **(E)** ELISA of 27H8 biotinylated antibody (27H8-biotin), IgG-biotin control, non-biotinylated 27H8 (27H8 w/o) and IgG1 Isotype control (IgG1 Isotype w/o) coated onto plates and detected by Streptavidin-HRP. For details, see Material and Methods section. **(F)** Biotinylated 27H8 antibody detected with Extravidin-AP was compared to unlabeled purified 27H8 antibody (w/o) detected by anti-IgG-AP. Further abbreviations **(A-F)**: Ggta1, α-galactosyltransferase; KO, knockout; OVA, Ovalbumin; BSA, bovine serum albumin; Ig, Immunoglobulin; MSA, mouse serum albumin; Dig., digested; w/o, without; HRP, horseradish peroxidase; ctrl, control; SAv, streptavidin.

(Figure 1C) per dot. 0.125 µg of glycolipids (with or without EGCase I digestion) and 1 µl of the whole blood lysate from a blood type B donor were spotted per dot. Pig kidney and cell lysates of cultured pig cells (pre-digested or not), α-Gal-APN, APN and thyroglobulin were spotted at an amount of 1 µg. Whole bacterial lysates were spotted at 1 µl without protein amount normalization. After a drying time of 15 min, the membrane was transferred to a chamber of mini-incubation trays (Bio-Rad Laboratories, Hercules, CA, USA) and blocked with 1.5 ml 2% BSA (Albumin Fraction V, AppliChem) in Tris-buffered saline (TBS, 20 mM Tris, 150 mM NaCl, pH 7.6, both AppliChem) for 1 hour at RT. Primary antibodies and lectin

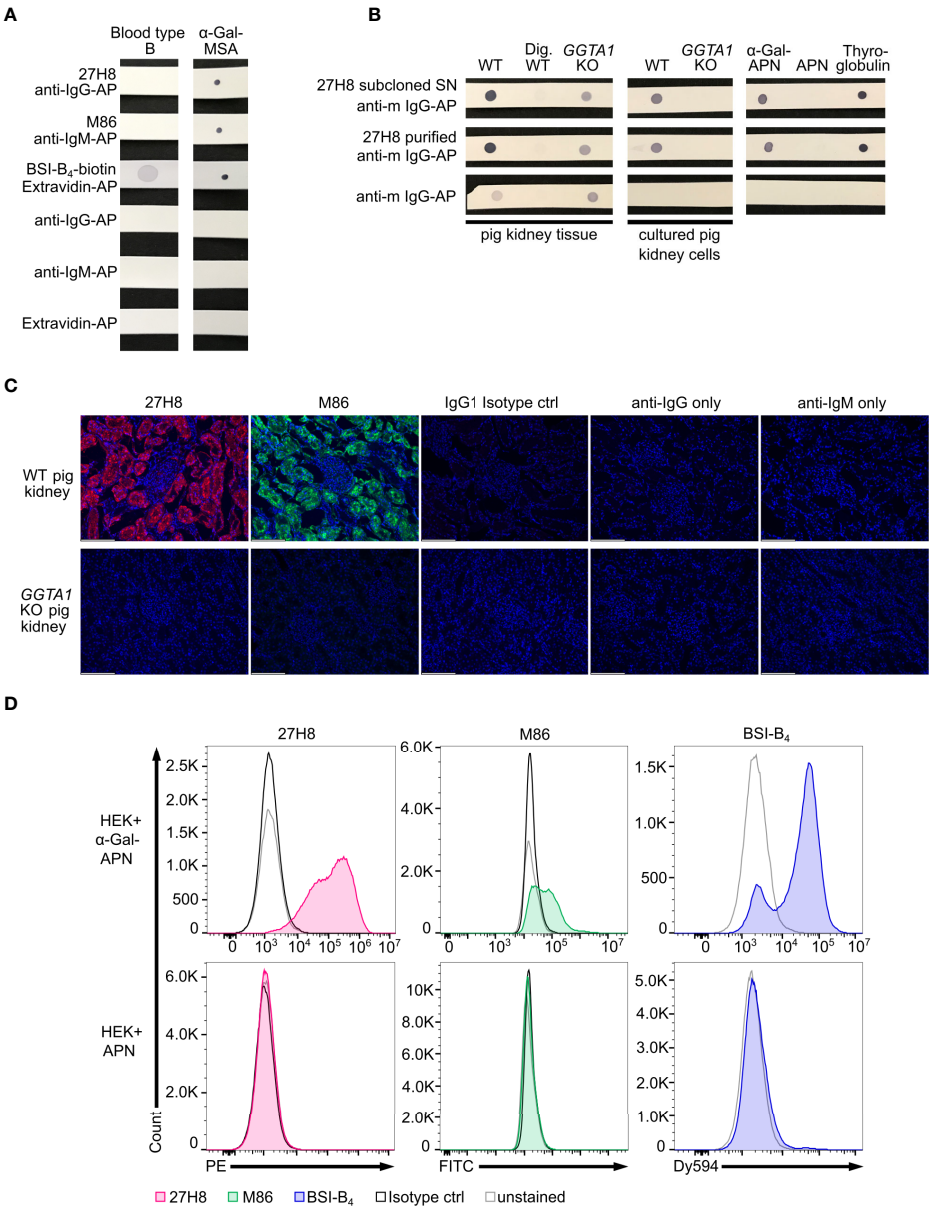

FIGURE 2
Specificity of 27H8 monoclonal antibody. **(A)** Dot blot stain of lysed whole blood from a type B blood donor and α-Gal-MSA (positive control) by 27H8, M86 or Lectin (BSI-B_4). **(B)** Screening of 27H8 subcloned hybridoma SN (upper row) and purified 27H8 antibody (middle row) on lysed kidney tissue or cultured kidney cells of wildtype (WT) and *GGTA1* knockout (KO) pigs and on WT kidney tissue samples digested with α-Galactosidase (Dig. WT). Further screening molecules: aminopeptidase N (APN) glycosylated with α-Gal, APN only and (α-Gal-containing) bovine thyroglobulin. **(A, B)** Samples in a row were blotted on one membrane. See Supplementary Figure 1 for uncropped blots. **(C)** Immunofluorescence microscopy of pig kidney tissue specimens (WT and *GGTA1* KO) stained with 27H8 (red) and M86 (green) in the glomerulus region. IgG1 isotype ctrl and secondary antibody only stains (anti-IgG1/anti-IgM) served as controls for fluorescence signal. DNA stained with DAPI (blue). Scale bar (white, left corner): 124.4 µm. **(D)** Flow cytometry analysis of human embryonic kidney (HEK) cells expressing α-Gal glycosylated APN (upper panel) and APN only (lower panel) stained with 27H8 (red), M86 (green) and BSI-B_4 (blue). Controls: unstained (grey) and Isotype controls (IgG1, IgM, both in black). **(A–D)** If not otherwise indicated, 27H8 was applied in the purified version.

were incubated over night at 4°C and diluted in 1 ml TBS supplemented with 1% BSA. Primary hybridoma supernatants from clones 27H8 and 25G8 were used at a 1:5 dilution in, purified and biotinylated 27H8 antibody at 0.6 µg/ml, M86 hybridoma supernatant (Enzo Life Sciences Farmingdale, NY, USA) in a 1:5 dilution and biotinylated lectin from *Bandeiraea simplicifolia* (BSI-B_4, Sigma-Aldrich) at 25 µg/ml. IgG isotype control (Invitrogen, Carlsbad, USA; polyclonal, Figure 4) and

IgG1 isotype control (Clone P3.4.2.8.1., Thermo Fisher Scientific, Figure 5B) for bacterial samples were used at 0.6 µg/ml. After primary detection, membranes were washed three times for 5 min with 1 ml 0.05% Tween20 (Calbiochem, Merck) in TBS (TBS/T). Secondary detection antibodies were incubated for 90 min at RT in 1 ml TBS/1%BSA. 27H8 primary hybridoma supernatant was detected by a monoclonal rat anti-mouse IgG1 (2E6, in house), 25G8 primary hybridoma supernatant was detected by a monoclonal rat anti-mouse IgG2b (7B8, in house) and in a tertiary incubation step with alkaline phosphatase (AP)-conjugated anti-rat IgG with minimal cross-reactivity (Jackson ImmunoResearch, Philadelphia, PA, USA) at a dilution of 1:5000. Both, supernatant from subcloned 27H8 and purified 27H8 antibody was detected by AP-conjugated anti-mouse IgG, Fc-specific (Sigma-Aldrich) at a dilution of 1:10,000. M86 was detected by AP-conjugated µ-chain specific anti-mouse IgM (Sigma-Aldrich) at a dilution of 1:30,000; BSI-B_4 and biotinylated 27H8 by AP-conjugated Extravidin (Sigma-Aldrich) at 1:10,000. Membranes were washed three times in TBS/T for 5 min and immersed in 0.01% nitro blue tetrazolium (AppliChem) and 0.005% 5-bromo-4-chloro-3-indolyl phosphate (AppliChem) in detection buffer (100mM Tris, 10 mM $MgCl_2$*$6H_2O$, 100 mM NaCl, pH 9.5) until dots were stained. After immersing membranes with distilled water, membranes were dried, aligned on a black paper and acquired with a photo camera at 15 cm height. For direct detection with horseradish peroxidase (HRP)-labeled secondary antibodies, glyco-/proteins or glycolipids were spotted on a membrane strip pre-wet in transfer buffer containing 25 mM Tris, 19.2 mM glycin and 20% isopropanol, pH 8.3. Rat anti-mouse-IgG1 (2E6, in house) labeled with HRP (for 27H8 primary supernatant) and rat anti-mouse-anti-IgG2b-HRP (for 25G8 primary supernatant). Uncropped dot blots from all figures are displayed in Supplementary Figure 1.

2.10. Periodic acid treatment

Nitrocellulose membranes with blotted samples were incubated in 40 mM periodic acid (H_5IO_4, Merck) diluted from a stock concentration of 200 mM in 50 mM sodium acetate buffer (AppliChem, adjust to pH 4.5 with HCl) for 1 hour at RT.

2.11. Antibody biotinylation

Purified 27H8 antibody was labeled with biotin-7-NHS using a Biotin Protein Labeling Kit (Roche) at a molar ratio of 1:10. Excess biotin-7-NHS was removed by gel filtration according to manufacturer's instructions. To verify the biotinylation efficiency, biotinylated 27H8 and a random biotinylated IgG antibody (biotinylated rat anti-mouse IgM, clone R6-60.2, BD, Franklin Lakes, USA) and as controls 27H8 without biotinylation and IgG1 isotype control (Southern Biotech, Birmingham, AL, USA) were coated at 2 µg/ml on a flat bottom MaxiSorp 454 96-well plate (Thermo Fisher Scientific) in 50 µl/well in sodium carbonate-bicarbonate buffer (pH 9.5) for 16 hours. The plate was washed three times with PBS/T (0.05% Tween), blocked with 300 µl 1% BSA and washed again three times. 50 µl streptavidin-HRP (BD) diluted 1:250 was added for 1 hour. Plate was washed again eight times and 50 µl 3,3′,5,5′-tetramethylbenzidin (1-Step Ultra TMB ELISA, Thermo Fisher Scientific) was added. Reaction was stopped with 25 µl 2M H_2O_2 (Merck) and emission was measured at 450 nm using a plate reader (Epoch, Biotek, Thermo Fisher Scientific).

2.12. Immunohistochemistry

0.25 cm x 1 cm sections of WT and *GGTA1* KO pig kidneys were fixed in 3.6% buffered formaldehyde (Fischar, Saarbrücken, Germany) for 24 hours and embedded in paraffin. Sections of 4 µm were cut and transferred to slides. Slides were washed twice with Xylol (Carl Roth, Karlsruhe, Germany) for 10 min. For rehydration, slides were transferred into a graded series of ethanol in distilled water: 100% (twice, 5 min), 96% (5 min), 70% (5 min), 50% (1 min), H_2O (30 seconds) and washed for 5 min in PBS (Sigma-Aldrich). Antigen was retrieved by transferring slides into nearly boiling citrate buffer, incubating at 90°C (10 min) and slowly cooling to RT (~30 min). Slides were washed 5 min in PBS and blocked with 2% BSA in PBS for 1 hour. Tissue sections were incubated with primary antibody solutions in 1% BSA at the following concentrations/dilutions: 1 µg/ml 27H8 or IgG1κ isotype control (Clone: P3.6.2.8.1, unconjugated, eBioscience) or 1:5 dilution of M86 supernatant (IgM) for 16 hours at 4°C. Slides were washed three times with PBS for 5 min and incubated for 30 min at RT with fluorochrome labeled secondary antibody diluted in 1%BSA in PBS at the following concentrations: goat anti-mouse IgG (H+L) conjugated to Alexa Fluor 647 (polyclonal, Thermo Fisher Scientific) at 2 µg/ml and goat anti-mouse IgM (heavy chain) conjugated to Alexa Fluor 488 (polyclonal, Thermo Fisher Scientific) at 10 µg/ml. Slides were washed in PBS (three times, 5 min) and 1 drop of ProLong Diamond Antifade mounting medium with DAPI (Life Technologies by Thermo Fisher) was added. Images were acquired on a Leica DM4B fluorescence microscope and processed using LAS X software (Leica, Wetzlar, Germany) with a 20X objective. Contrast and brightness were adjusted simultaneously on all images per channel with ImageJ software (https://imagej.nih.gov/ij/, Rasband, W.S., U. S. National Institutes of Health, Bethesda, MD, USA). All antibody solutions were centrifuged to remove antibody complexes before use.

2.13. Eukaryotic flow cytometry

HEK cells expressing α-Gal glycosylated APN and APN devoid of α-Gal (see description in screening material above) were washed with BSA-Buffer containing 1% BSA in PBS. $5x10^5$ cells were seeded and stained with either 27H8 purified antibody or IgG1κ isotype control (clone: B3102E8, Southern Biotech) at 1 µg/ml, a 1:10 dilution of M86 supernatant, a 1:10 dilution (40 µg/ml) of mouse IgM isotype control (clone: MOPC 104E, Sigma) or a 1:100 dilution of BSI-B_4 conjugated to DyLight®594 (*Griffonia simplicifolia* isolectin B4, Vector Laboratories, Burlingame, CA, USA) in BSA-buffer. Cells were washed twice and stained with respective secondary antibodies: anti-mouse IgG1-PE (clone: A85-1, BD Pharmingen) at a 1:100 dilution or anti-mouse IgM-Alexa Fluor® 488 (clone: 1B4B1, Southern Biotech) at a 1:500 dilution. Staining was performed in 100 µl for both primary and secondary antibody incubation steps for 45 min. Cells were washed twice and resuspended in 150 µl BSA-buffer before acquisition at a Novocyte Quanteon Flow Cytometer.

For murine splenocyte staining, spleen was excised and meshed on a 70 µM filter for generating a single cell suspension. After washing splenocytes twice with PBS, erythrocytes were lysed with an ACK lysis buffer (155 mM NH_4, 10 mM $KHCO_3$, 0.1 mM EDTA-2Na-$2H_2O$; pH 7.2-7.4) in 1 ml for 2 min. Cells were washed with BSA-SA-buffer twice before staining. Staining and acquisition were performed as described for intestinal bacterial staining (see protocol below), except that splenocytes were stained with 5 µg/ml 7-Aminoactinomycin D (7AAD, Enzo Life Sciences) instead of SYBR green identification of living cells (Supplementary Figure 2B).

2.14. Bacterial flow cytometry

Bacteria were grown to a density of 10^9 CFU/ml. *S. aureus* 20231 and *E.coli* K12 were grown overnight at 37°C in L.B. medium. 100 µl of the culture was seeded into a 96-well U-bottom plate and washed with BSA-SA-buffer containing 1% BSA, 0.05% sodium azide (Morphisto, Offenbach am Main, Germany) in PBS at 4000xg for 5 min. Cells were stained with 1 µg/ml 27H8 and washed twice and stained with anti-IgG1-PE antibody (clone A85-1, BD, Franklin Lakes, NJ, USA) at a 1:100 dilution in a total volume of 50 µl. Bacteria were washed twice and fixed for 30 min with 3.7% formaldehyde (AppliChem, 37% diluted 1:10 in PBS) and washed again twice before acquisition in 100 µl PBS at an Acurri™ Flow Cytometer (BD). *E. coli* O86: B7, BL21, HS and *L. rhamnosus* were cultivated o.n. at 37°C in 5 ml L.B. medium shaking at 150 rpm. Cells were centrifuged (4000xg, 5 min) and washed twice with PBS before fixing cells in 4% PFA for 30 min. Bacteria were washed with PBS and then stained with primary and secondary antibody as described for HEK cells (see protocol above). Bacterial pellet was resuspended in 100 µl BSA-buffer for acquisition on a Novocyte Quanteon Flow Cytometer. In general, at least 5 x 10^5 events were acquired.

For intestinal bacteria staining of *Ggta1* KO and WT mice, the entire small intestine, cecum and colon were removed. Small intestine was cut longitudinally and whole content streaked out with a sterile pipette tip into a 1.5 ml tube. The cecum was cut on the tip and 2/3rds of the content streaked out. For the colon, the whole content was streaked out. 1 ml BSA-SA-buffer was added and slurry mixed by vortexing and pipetting. Intestinal debris was spun down at 900xg for 5 min, 4°C, and supernatant was transferred to a new tube for another centrifugation step at 450xg for 5 min, 4°C, to remove host cells. Bacterial pellets were washed twice in 1 ml BSA-SA-buffer at 8000xg 5 min, 4°C and filtered (70 µM) before seeding 100 µl of washed small intestine content, 25 µl of cecum content, 50 µl of colon content into U-bottom plates. Pellets were centrifuged, supernatant removed and stained in 50 µl for 30 min. Concentrations and dilutions were: 1 µg/ml biotinylated 27H8 or IgG1κ isotype control (clone P3.6.2.8.1., biotinylated, eBioscience/Thermo Fisher Scientific) or 1:40 dilution of biotinylated BSI-B_4. Before staining with a 1:500 dilution of streptavidin-PE (SAv-PE, BD) cells were washed twice by a centrifugation step at 3200xg for 5 min at 4°C. After two additional washing steps, bacteria were resuspended in 200 µl of a 1:100,000 dilution of SYBR green (SYBR green I nucleic acid gel stain, Sigma-Aldrich), incubated for 5 min and acquired at an Acurri™ Flow Cytometer. Data analysis of FCS-files was performed with FlowJo (Version 10.7.1) and SYBR green positive were considered as bacteria (Supplementary Figure 2A) as described in (34).

2.15. Surface Plasmon Resonance analysis

The binding measurements were performed on a BIACORE 3000 instrument (Biacore Inc., Piscataway, NJ, USA) and analyzed with Origin software version 9.0. 27H8 purified antibody was diluted to a final concentration of 50 nM in 10 mM sodium acetate, pH 4.0, and chemically immobilized (amine coupling, 850 RU bound) onto CM5 sensor chip (Cytiva). α-Gal-DI-BSA and α-Gal-TRI-BSA were diluted in the running buffer (PBS, 1 mM DTT and 0.005% Tween 20) to the final concentration of 0.977 nM, 1.95 nM, 3.91 nM, 7.81 nM, 15.6 nM, 31.3 nM, 62.5 nM, 125 nM, 250 nM, 500 nM and injected over the sensor chip surface at 30 µL/min at 25°C. The protein samples were injected onto the sensor chip from the lowest to the highest concentration. Both glycoprotein samples were tested three times. Injection of 250 nM ligand was performed in duplicate within each experiment. In order to subtract background noise from each experiment, all samples were run over an unmodified CM5 sensor chip surface. After each ligand injection, the sensor chip was regenerated using 3 M $MgCl_2$

solution. For each measurement the equilibrium dissociation constant was calculated (K_D). The K_Ds from three experiments were used to calculate the mean values of these variables and the standard deviation.

2.16. Enzyme-linked Immunosorbent Assay

For comparing supernatant from subcloned 27H8 hybridoma and M86 hybridoma supernatant, both antibodies were titrated on glycoproteins coated to standard ELISA plates. α-Gal-DI-BSA, α-Gal-TRI-BSA, α-Gal-OVA, α-Gal-MSA and respective negative control proteins BSA, OVA and MSA were coated at a concentration of 5 µg/ml in 50 µl per well on a flat bottom Maxi-Sorp 96-well plate (Thermo Fisher Scientific) for 12 hours at 4°C in sodium carbonate-bicarbonate buffer, pH 9.5. Plates were washed three times with PBS/T and blocked with BSA-buffer for 1 hour at RT and washed again three times with PBS/T. 27H8 supernatant and M86 were titrated in BSA-buffer starting from 1.12 µg/ml in a serial 1:10 dilution to 1.12 ng/ml. The starting concentration was set according to the stock concentration of the M86 antibody in the hybridoma supernatant. The amount of 27H8 and M86 antibody in the hybridoma supernatants was measured with a Biotech Clonotyping System-HRP Kit and mouse Immunoglobulin Panel for Standards (both Southern Biotech) according to the manufacturer's instruction, yielding a concentration of 116.69 µg/ml 27H8 antibody in the supernatant and 1.12 µg/ml of M86. IgG1 and IgM Isotype controls (Southern Biotech) were used at the highest concentration at 1.12 µg/ml. Primary antibodies incubated for 1 h at RT and plates were washed 5x with PBS/T. Polyclonal antibody conjugated to horseradish peroxidase (HRP) detecting both mouse IgG and IgM heavy and light chains (Jackson ImmunoResearch, West Grove, PA, USA) were incubated at a concentration of 80 pg/ml (1:10,000 dilution) in 1% BSA in PBS in 50 µl per well for 1 hour at RT shaking at 450 rpm. Plates were washed again 8 times with PBS/T and 50 µl TMB substrate (1-step Ultra TMB, Thermo Fisher Scientific) was added before stopping the reaction with 25 µl 2M sulfuric acid (Merck). Emission was measured with a plate reader at 450 nm. ELISAs were repeated three times. Analysis, logarithmic transformation and curve fit (nonlinear variable slope, 4 parameters) was performed with GraphPad Prism 7 (GraphPad Software Inc.)

For epitope blocking ELISAs, α-Gal MSA was coated onto plates at 0.5 µg/ml in 50 µl per well as described before. Plates were washed with PBS/T, blocked with BSA-buffer and washed again as described before. Blocking antibody 27H8 supernatant was added in a serial dilution (1:10) from 100 µg/ml to 0.01 µg/ml in BSA-buffer. As the concentration of M86 was low compared to 27H8 in supernatant, a serial dilution of M86 was applied from 1 µg/ml to 0.01 µg/ml. The blocking antibody was incubated for 1 hour at RT with shaking at 500 rpm and plates were washed 5 times. Afterwards, the competing antibody (27H8 supernatant for M86 block and M86 for 27H8 supernatant block) was incubated for 1 hour at a concentration of 0.1 µg/ml at RT and shaking at 500 rpm and wells were washed 5 times. Detection was performed with either anti-IgG1-HRP for 27H8 competing antibody or anti-IgM-HRP for M86 competing antibody (both from Southern Biotech) at a 1:500 dilution. TMB substrate addition and acquisition were done as described before.

For measurement of human IgG, IgM and IgE antibodies from serum, bovine thyroglobulin (Sigma Aldrich) was coupled onto plates as described above. After washing, plates were blocked with chicken serum albumin (Sigma Aldrich). Diluted serum was added and incubated for 2 hours at RT. After washing, the biotinylated primary antibody specific for the indicated isotypes was incubated for 1 hour at RT. Detection was performed using streptavidin-HRP and acquisition was done as described before using TMB substrate.

2.17. Statistical analysis

For statistical analysis, a one-way ANOVA with Tukey's multiple comparisons test was performed with GraphPad Prism 7 (GraphPad Software Inc.). A p value of $p < 0.05$ was considered statistically significant.

3. Results

3.1. The novel 27H8 monoclonal antibody specifically binds to α-Gal epitopes

In order to generate a monoclonal antibody specific for the α-Gal epitope determining structure Gal-α1,3-Gal that is equally able to bind to the naturally occurring α-Gal epitope Gal-α1,3-Gal-β1,4-GlcNAc, we immunized α-galactosyltransferase knockout mice (*Ggta1* KO) (26) with Gal-α1,3-Gal-β1,4-GlcNAc coupled to ovalbumin as carrier protein (α-Gal-OVA) according to the scheme depicted in Figure 1A. Splenic B cells were fused with the myeloma cell line P3X63-Ag8.653 and primary hybridoma supernatants were screened for IgG antibodies binding to Gal-α1,3-Gal-bovine serum albumin (α-Gal-DI-BSA) in a flow cytometric bead assay (Figure 1A). Screening for antibodies against Gal-α1,3-Gal coupled to a different carrier protein than used for immunization minimized the risk of pulling out antibody clones specific to the immunization molecule OVA. To further diversify immunization and screening molecule and avoid off-target (linker) specific antibodies, different linker lengths were selected with a 14-C-atom linker for the immunization molecule α-Gal-OVA and a 3-C-atom-linker for the screening molecule α-Gal-

DI-BSA. Overall, 1536 supernatants from 4 immunized mice were screened and only two primary hybridoma supernatants (25G8 and 27H8) showed binding to α-Gal-DI-BSA. The determined isotype in the 25G8 primary hybridoma supernatant was IgG2b kappa, that of 27H8 IgG1 kappa. In a secondary screen, α-Gal-conjugated glycoproteins and respective control proteins without α-Gal were spotted on a membrane (dot blot) and incubated with either rat anti-mouse IgG1 (for 27H8) or rat anti-mouse IgG2b (for 25G8) and detected with anti-rat antibodies (Figure 1B). Both 25G8 and 27H8 primary hybridoma supernatants bound to α-Gal-conjugated mouse serum albumin (MSA), α-Gal-OVA and the disaccharide and trisaccharide α-Gal epitopes conjugated to BSA (α-Gal-DI-BSA/α-Gal-TRI-BSA). While 25G8 also strongly detected the carrier molecule OVA that was used for immunization, 27H8 showed only minimal binding to OVA (Figure 1B, upper two rows). Both primary hybridoma supernatants bound to glycolipids isolated from rabbit erythrocyte membranes rich in α-Gal (30). Binding was prevented by cleaving the carbohydrate from the lipid through pre-incubation with endoglycoceramidase I (EGCaseI) (Figure 1B), an enzyme hydrolysing the β-glycosidic covalent link between oligosaccharide and ceramide. In a second screening assay using a wet membrane and horseradish peroxidase (HRP)-labeled secondary antibodies, binding of 25G8 to OVA was still visible while binding of 27H8 was not detectable at all (Figure 1C). Therefore, 27H8 hybridoma cells were chosen for subcloning by limiting dilution to generate a stable monoclonal hybridoma cell line. Antibodies were purified from monoclonal 27H8 supernatant with protein A and both, supernatant and purified 27H8 antibody were validated alongside in a secondary dot blot screening with direct detection using an alkaline phosphatase (AP)-conjugated anti-mouse IgG antibody (Figure 1D). Both, the supernatant and purified 27H8 antibody showed a highly specific binding to all tested α-Gal carrying glycoproteins and -lipids but did not show any binding to OVA (Figure 1D). Thus, the initially observed weak binding of the primary 27H8 supernatant to OVA (Figure 1B) was most likely caused by a second hybridoma clone growing in the same well as 27H8, as in the first screening round monoclonality cannot be assumed. Next, the purified 27H8 antibody was conjugated to biotin. Successful biotinylation was validated in an enzyme-linked immuno assay (ELISA) by coating the biotinylated 27H8 as well as a biotinylated control antibody on plates followed by detection with streptavidin conjugated to HRP. The antibody 27H8 could be labeled with a similar efficiency as the control antibody (Figure 1E). The biotinylated 27H8 antibody in combination with Extravidin-AP showed a highly specific α-Gal detection without any detectable background staining to carrier molecules devoid of α-Gal (Figure 1F). In summary, the newly generated 27H8 monoclonal antibody binds to both the di- and trisaccharide epitope of α-Gal irrespective of its conjugation to proteins or lipids, it can be easily purified by protein A chromatography and can be labeled with biotin for enhanced detection and applicability.

3.2. 27H8 monoclonal antibody detects α-Gal epitopes of natural origin and offers a wide range of possible applications

To verify the specificity, the 27H8 monoclonal antibody was compared to *Bandereia simplifolica* isolectin B_4 (BSI-B_4) and to the monoclonal IgM antibody M86, which are both widely used to detect the α-Gal epitope (2, 18, 24). BSI-B_4 is specific for terminal α-galactose oligosaccharides (35) and therefore recognizes also the blood group B antigen, which differs from the α-Gal epitope only in the addition of one fucose residue and is thus structurally very similar (36, 37). To assess whether 27H8 also binds to the blood group B antigen we blotted lysates of whole blood from a type B donor on a membrane and applied the antibodies 27H8 and M86 or biotinylated BSI-B_4 for detection. While BSI-B_4 bound to the blood type B specimen as expected, neither 27H8 or M86 did (Figure 2A). Next, we investigated whether 27H8 also binds to natural α-Gal epitopes. As pig kidney is naturally rich in α-Gal (38, 39) and reactions in α-Gal allergic patients are severe after ingestion (9), we tested if 27H8 recognizes α-Gal in pig kidney lysates in a dot blot assay. 27H8 binding to wildtype (WT) pig kidney lysate was observed with strong staining intensity (Figure 2B left panel). Control staining with the secondary anti-mouse IgG-AP antibody gave a faint signal on WT pig kidney lysate as well as on *GGTA1* KO cells lysates without or after 27H8 staining. However, no cross-reactivity of the secondary antibody was observed in WT pig kidney tissue lysates digested with α-galactosidase, an enzyme that cleaves off terminal α-galactose (40), indicating a relevance of galactose glycosylation for background staining by the secondary antibody. To avoid this background staining, we tested 27H8 on lysates from cultured pig kidney cells devoid of pig immunoglobulins. Here, background staining was not observed for anti-mouse IgG-AP on lysates from cultured cells and 27H8 bound exclusively to WT cultured pig kidney cells but not to *GGTA1* KO cultured pig kidney cells (Figure 2B middle panel). This result suggests that the secondary antibody used for detection still recognizes pig IgG antibodies present in whole kidney lysate despite anti-mouse-IgG-AP being highly cross-absorbed against immunoglobulins from various species. Additionally, we tested 27H8 on purified aminopeptidase N (APN) from HEK cells either expressing the α-1,3-galactosyltransferase or not. 27H8 only bound to α-Gal-APN and not to APN (Figure 2B right), further verifying its specificity to the α-Gal epitope. Importantly, 27H8 also recognizes bovine thyroglobulin – a protein used for α-Gal specific IgE antibody detection assays for red meat allergy patients [ImmunoCAP, Thermo Fisher Scientific, also described in (41)] (Figure 2B right). Specific binding of 27H8 to WT but not to *GGTA1* KO pig kidney was also observed on tissue slides using a monoclonal secondary antibody in

immunohistochemistry (Figure 2C). 27H8 bound to the same cellular structures as M86 (Figure 2C upper left), such as binding to cells of the nephron's tubular system but not to the glomerulus. Flow cytometry analysis of HEK cells expressing α-Gal-APN and APN confirmed specificity of 27H8 to natural α-Gal epitopes and highlights the broad range of applications of this antibody for detection of α-Gal epitopes in dot blot, histology and flow cytometry (Figure 2D). We therefore conclude that the 27H8 monoclonal antibody is highly specific for the α-Gal epitope in natural settings, does not bind the blood type B antigen and offers a wide range of possibilities for application

3.3. 27H8 monoclonal antibody binds with high affinity to α-Gal epitopes and competes with M86 for recognition

After determining the specificity and applicability of 27H8, we aimed to evaluate binding affinities of 27H8 antibody for the di- and trisaccharide α-Gal epitopes in a quantitative manner, and performed Surface Plasmon Resonance (SPR) analyses with both α-Gal-DI-BSA and α-Gal-TRI-BSA molecules (Figure 3A). Both analytes bound in a nanomolar concentration range to the coupled 27H8 antibody. The mean dissociation constant (K_D) was slightly higher for α-Gal-TRI-BSA (7.51 ± 1.9) than for α-Gal-DI-BSA (2.02 ± 1.0), indicating a higher affinity of 27H8 for the disaccharide than the trisaccharide epitope. However, this might be partly explained by 35 sugar residues being attached to one molecule BSA for the α-Gal-DI-BSA analyte, while α-Gal-TRI-BSA consists of 33 sugar residues on average per protein. The Hill coefficients for the fitted binding curves of both analytes is smaller than one ($n < 1$), indicating negative cooperativity between the binding sites on 27H8 antibody (Figure 3A). Negative cooperativity suggests that the first binding analyte (α-Gal-DI-BSA or α-Gal-TRI-BSA) decreases the rate of subsequent analyte binding. As a full-length IgG antibody has two identical antigen-binding sites and due to the size of the BSA conjugated analytes (66kDa + 33 or 35 sugar residues), we assume that binding of one α-Gal-DI/TRI-BSA molecule to the first binding site on the 27H8 antibody may partially block the access of the second α-Gal-DI/TRI-BSA molecule to the second antigen-binding site as a result of steric hindrance. We next sought to compare the 27H8 antibody to M86, the most widely used monoclonal antibody specific for the α-Gal epitope developed by Galili *et al.* (24). This IgM antibody is commonly available as a hybridoma supernatant but for a direct affinity comparison both antibodies are ideally used in a purified format. However, while 27H8 antibody can easily be purified with protein A (Figure 1D), we were unable to purify M86 antibody with commonly used purification reagents such as recombinant protein L (Cytiva Capto™ L, Thermo Fisher Scientific, data not shown). Thus, we compared the hybridoma supernatants of 27H8 and M86 regarding their respective binding to α-Gal conjugated glycoproteins in an ELISA (Figure 3B). In order to titrate both antibodies to equal concentrations, we determined the antibody amount in the supernatants by interpolating OD 450 nm values to a standard curve of IgG1 and IgM isotype controls by a standard immunoglobulin isotype ELISA. To analyze the values in the linear range of the standard curve and dynamic range of the assay, 27H8 supernatant and M86 supernatant had to be diluted to variable degrees, which decreases the accuracy of concentration measurements. Thus, the concentrations of immunoglobulins in 27H8 supernatant and M86 supernatant are estimates. Additionally, antibody concentration in the 27H8 supernatant stock was approximately 100 times higher than in the M86 supernatant (27H8 supernatant: ~116.69 µg/ml; M86 supernatant: ~1.12 µg/ml) (see Material and Methods). When comparing 27H8 supernatant and M86 supernatant titration curves on α-Gal-DI-BSA and α-Gal-TRI-BSA coated to ELISA-plates, we observed that both antibodies bind the di- and trisaccharide epitopes of α-Gal with a similar avidity (Figure 3B upper panel). This similar binding property was also observed on α-Gal-OVA- (Figure 3B middle panel) and α-Gal-MSA-coated plates (Figure 3B lower panel). Supernatant of subcloned monoclonal 27H8 hybridoma did not bind respective proteins devoid of α-Gal, such as BSA, MSA and, importantly, it did not bind OVA (Figure 3B right), in contrast to the weak binding of the primary hybridoma supernatant to OVA (Figure 1B). To further confirm that 27H8 and M86 recognize the α-Gal epitope in a similar manner we performed blocking assays in which the antibodies competed with each other for α-Gal binding (Figure 3C). α-Gal-MSA was coated onto ELISA-plates and incubated with increasing amounts of either 27H8 supernatant or M86 supernatant in a serial dilution to block the α-Gal epitope. The maximum concentration of M86 used for blocking was limited to 1 µg/ml due to the low stock concentration, while 27H8 supernatant was increased up to 100 µg/ml. Afterwards, the respective competing antibody was added (27H8 to M86 block and M86 to 27H8 block) and detected with specific anti-IgG1 or anti-IgM antibodies, respectively. 27H8 supernatant binding was blocked by M86 at concentrations higher than 0.1 µg/ml while 27H8 supernatant blocked M86 binding gradually even at lower amounts (starting from ~0.01 µg/ml). This discrepancy might be explained by the different isotypes (IgG1 vs IgM) and steric inhibition by IgM pentamers, but also confirms the high affinity of 27H8 monoclonal IgG1 antibody to the α-Gal epitope. Furthermore, we confirmed that 27H8 has a different variable domain sequence in the CDR regions compared to M86 which translates also in a different amino acid sequence and thus epitope recognition [(42) and data not shown]. In brief, the novel 27H8 antibody binds the α-Gal epitope comparable to M86 in ELISA and displays high affinity for its epitope.

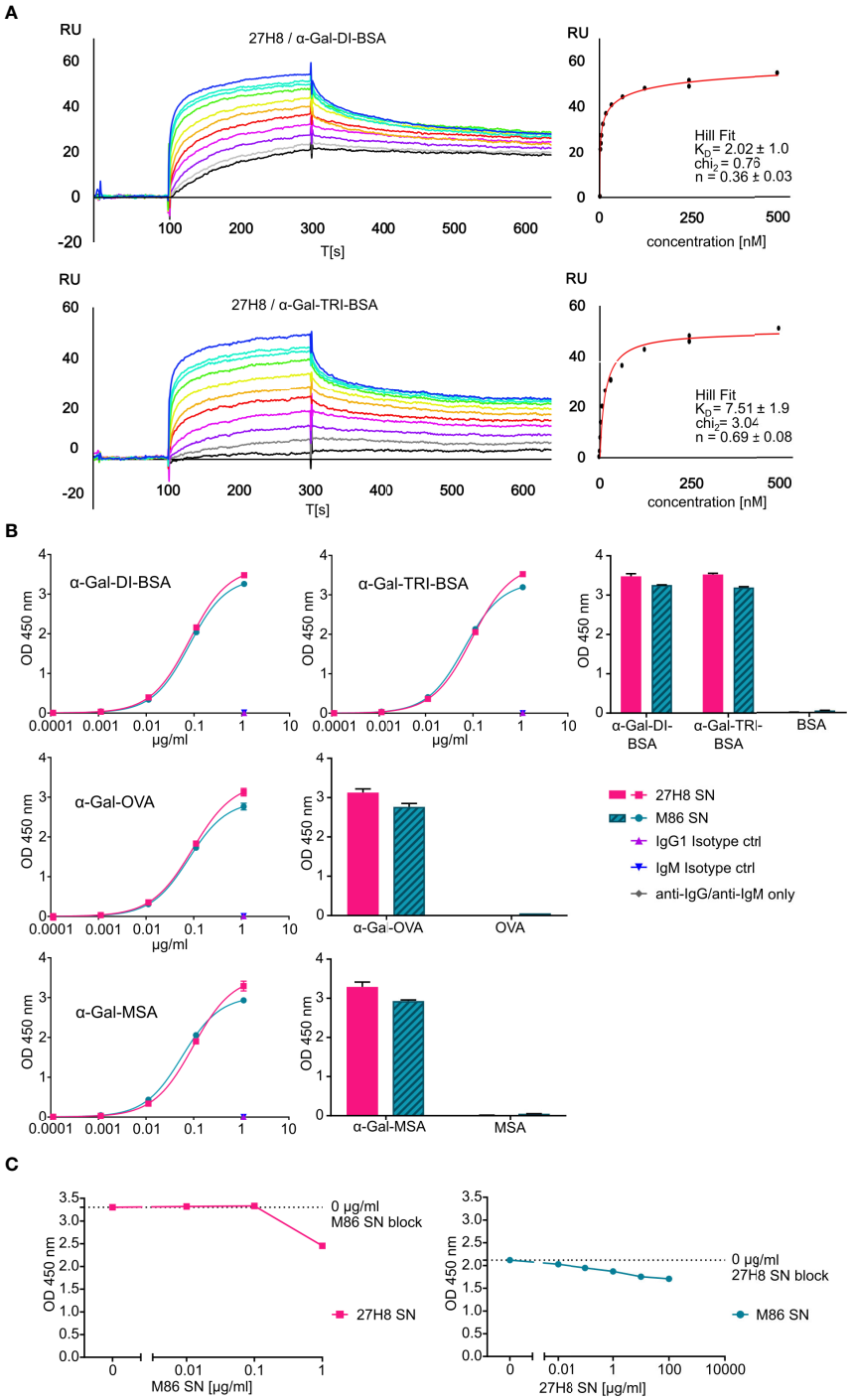

FIGURE 3
High affinity recognition of synthetic α-Gal epitopes. **(A)** Graphs show binding of synthetic α-Gal ligands to the coupled purified 27H8 antibody *via* surface plasmon resonance (SPR). Representative sensorgrams are displayed for 27H8/α-Gal-DI-BSA (upper panel) and 27H8/α-Gal-TRI-BSA (lower panel). For both pairs calculated equilibrium dissociation rate (K_D), error as standard deviation from three independent experiments, the chi^2 value for the curve fit and the Hill coefficient n are shown (right panel). Concentration series color code: black: 0.98 nM, gray: 1.95 nM, violet: 3.9 nM, magenta: 7.8 nM, red: 15.6 nM, orange: 31.2 nM, yellow: 62.5 nM, green: 125 nM, cyan: 250 nM, blue: 500 nM. RU: response units, T[s]: time in seconds. **(B)** Titration of 27H8 subcloned hybridoma SN and M86 hybridoma SN in ELISA on α-Gal conjugated glycoproteins (left) and direct comparison of glycoprotein and respective protein devoid of α-Gal at the highest concentration of 27H8 SN or M86 SN (1.12 µg/ml) (right). **(C)** Epitope blocking of α-Gal-MSA coated to ELISA-plates: M86 SN block followed by 27H8 SN and anti-IgG1 detection (left panel) or 27H8 SN block by M86 SN and anti-IgM detection (right panel). **(B, C)** Concentration values (x-axis) are plotted in logarithmic scale; antibody binding is shown as OD 450 nm (y-axis).

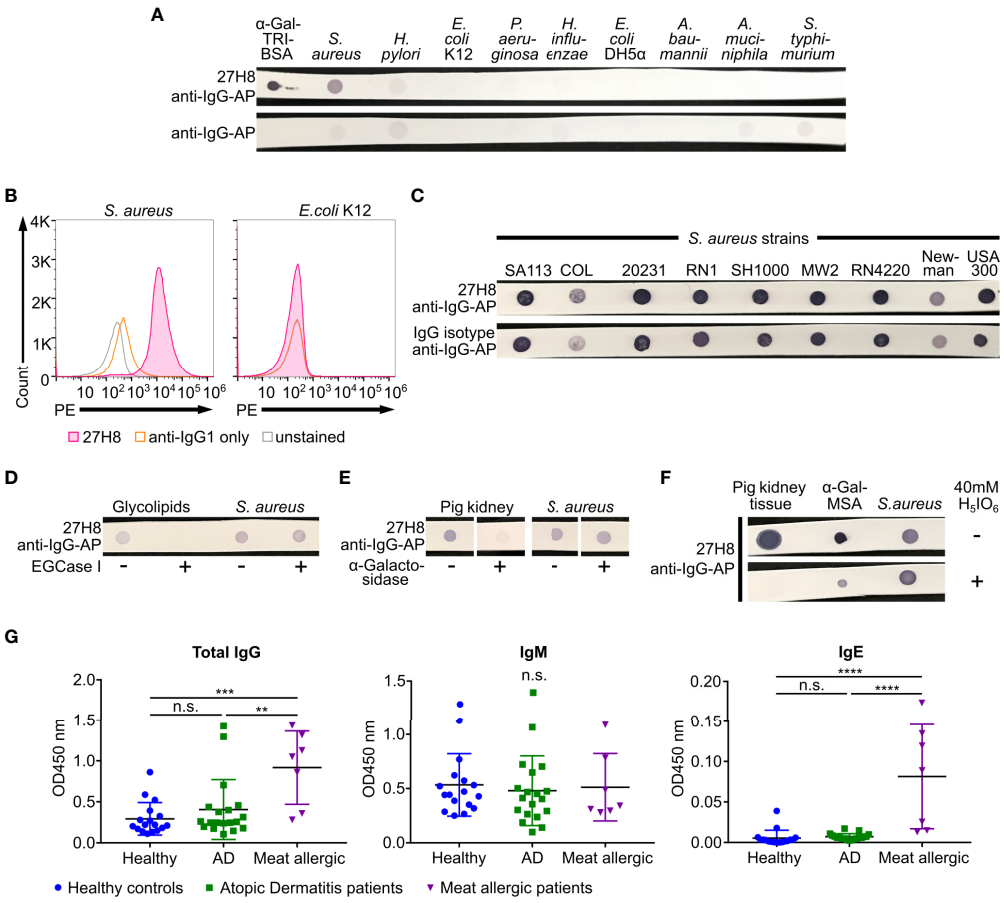

FIGURE 4
Staphylococcus aureus strains bind 27H8 independent of α-Gal expression. **(A)** Dot blots of the positive control α-Gal-TRI-BSA and lysed bacterial species *Staphylococcus aureus* (*S. aureus*, strain Mu50), Helicobacter pylori (*H. pylori*), *Escherichia coli* (*E. coli*, K12 and DH5α), *Pseudomonas aeruginosa* (*P. aeruginosa*), *Haemophilus influenzea* (*H. influenzae*), *Acinetobacter baumannii* (*A. baumannii*), *Akkermansia muciniphila* (*A. muciniphila*) and *Salmonella typhimurium* (*S. typhimurium*). **(B)** Histograms of flow cytometric analysis of *S. aureus* strain 20231 and *E.coli* K12 stained with 27H8 and anti-IgG1-PE. **(C)** Multiple *S. aureus* strains stained with 27H8 and IgG isotype in dot blot. **(D)** Dot blot of glycolipids and *S. aureus* strain SH1000 digested or not with EGCase I as indicated. **(E)** Dot blot of pig kidney lysate and *S. aureus* strain SH1000 digested or not with α-Galactosidase as indicated. Uncropped blots depicted in Supplementary Figure 1D. **(F)** Pig kidney lysate, α-Gal-MSA and *S. aureus* strain 20231 blotted on membrane and either incubated with Periodic acid (H_5IO_6) or not. **(A, D–F)** Detection with 27H8 and anti-IgG-AP. **(A-F)** 27H8 was applied in the purified version. **(G)** ELISA of human IgG, IgM and IgE binding to thyroglobulin in serum samples from healthy controls, Atopic Dermatitis (AD) or red meat allergic patients. Each symbol represents an individual subject. Statistics: one-way ANOVA with Tukey's multiple comparisons test, **p <0.01, ***p < 0.001, ****p < 0.0001, n.s.: not significant.

3.4. *Staphylococcus aureus* does not express the α-Gal epitope

Intestinal bacteria have been hypothesized to induce anti-α-Gal immunoglobulins (IgM, IgG) in humans (16). Thus, we investigated whether 27H8 antibody binds to bacteria reported to express α-1,3-galactosyltransferase-like genes (KEGG orthology number KO3275 or KO3278) as described by Montassier *et al.* (20), such as *H. pylori* (J99), *H. influenzae* (Hi375), *S. typhimurium* (ATCC 14028), *P. aeruginosa* (DSM 50071), *A. baumannii* (ATCC 17978) and *A. muciniphila* (ATCC BAA-835). Negative controls were selected according to literature, such as *E. coli* K12 (18). We further included *E. coli* DH5α and strains from the gram positive bacterium *S. aureus*, though it was reported that most α-Gal expressing bacteria were supposed to be gram-negative (20). Surprisingly, none of the tested bacterial lysates could be stained with the 27H8 antibody in a dot blot experiment, except *S. aureus* Mu50 and as positive control α-Gal-TRI-BSA (Figure 4A). The binding of 27H8 to *S. aureus* was not only observed in a dot blot but also by bacterial flow cytometry (Figure 4B). The fluorescence intensity increased in the secondary antibody only sample (anti-IgG1-PE) relative to the unstained control indicating a substantial background stain (Figure 4B, left panel). However, the first IgG1 isotype control we used (Southern Biotech) did not give the same fluorescence signal as 27H8 antibody when applying the same concentration

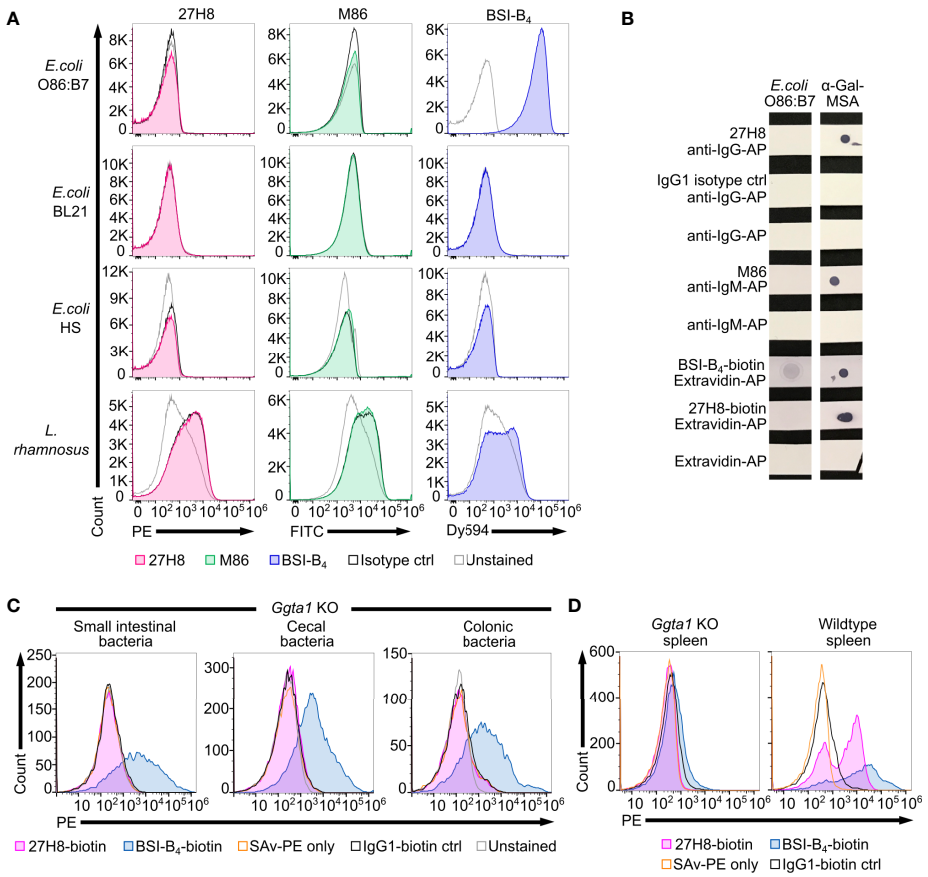

FIGURE 5
No binding of 27H8 and M86 to intestinal bacteria in contrast to BSI-B_4. **(A)** Histograms of flow cytometric analysis of cultured bacterial strains stained with 27H8, M86 and BSI-B_4 and respective isotype controls (IgG1 and IgM). Strains: *E coli* O86:B7, *E.coli* BL21, *E coli* Human Species (HS) and *Lactobacillus rhamnosus* (*L. rhamnosus*). **(B)** Dot blot stain of lysed *E coli* O86:B7 and α-Gal-MSA as positive control. Uncropped blots depicted in Supplementary Figure 1E. **(C)** Representative histogram blots of flow cytometric analysis of intestinal content (derived from small intestine, cecum and colon) from *Ggta1* KO mice (n=3) stained with biotinylated 27H8 and BSI-B_4, pre-gated for SYBR green positive bacteria (Supplementary Figure 2A). **(D)** Live splenocytes of *Ggta1* KO and WT mouse (Supplementary Figure 2B) stained with biotinylated 27H8 and BSI-B_4. **(C, D)** Control staining with IgG1-biotin isotype and streptavidin-PE (SAv-PE) only.

(data not shown). We further observed that 27H8 binding to *S. aureus* is a shared pattern for multiple strains but if we applied a polyclonal IgG isotype control, the same staining intensity was observed as for 27H8 (Figure 4C). This result suggests that the binding of 27H8 to *S. aureus* strains is likely a common feature of IgG antibodies regardless of specificity and is not due to a specific binding to the α-Gal epitope present on this bacterium. To further demonstrate that *S. aureus* does indeed not express α-Gal, we cleaved and removed the α-Gal epitope by various approaches and examined 27H8 binding thereafter. First, we removed the α-Gal carrying oligosaccharide in the *S. aureus* sample by EGCase I digestion, but in contrast to control digestion of glycolipids, no signal was lost for *S. aureus* (Figure 4D). Furthermore, when comparing pig kidney tissue lysate and *S. aureus* lysate digested with α-Galactosidase, we observed a significant signal reduction for the mammalian sample, but not for the bacterial sample (Figure 4E). Finally, when the membrane of blotted samples of pig kidney tissue lysate, α-Gal-MSA and *S. aureus* lysate was pre-incubated with periodic acid, a treatment that destroys all carbohydrate determinants (43), the staining intensity of 27H8 was lost or substantially reduced for pig kidney and α-Gal MSA, but not for the *S. aureus* sample (Figure 4F). Thus, the 27H8 antibody binds to a structure in *S. aureus* that is not part of an oligosaccharide connected to a sphingolipid, does not contain α-galactose residues and is not even a carbohydrate. Most probably, 27H8 binds to protein A, as already implied for human polyclonal anti-α-Gal antibodies binding to *S. aureus* human isolates (17). In line with this result, we could not detect any staining of *S. aureus* samples with M86 in a dot blot as IgM antibodies are typically not bound by protein A (data not shown). Furthermore, and in contrast to meat allergic patients, we did

not observe enhanced IgG or IgE titers against bovine thyroglobulin [a molecule routinely used to detect anti-α-Gal antibodies in patient serum samples (41)] in our selection of atopic dermatitis patients (Figure 4G). We selected this patient group as atopic dermatitis patients are usually strongly colonized by *S. aureus* (44). Serum IgM titers against thyroglobulin were unchanged between the groups. Altogether, these data strongly indicate that *S. aureus* binds *via* protein A to the constant part of the 27H8 antibody and does not express α-Gal itself.

3.5. 27H8 and M86 antibodies do not bind to *E. coli* O86:B7 nor to other members of the intestinal microbiota

As we did not observe any binding of 27H8 antibody to lysates of cultivated bacteria in a dot blot (Figure 4A), we next sought to test for binding of 27H8 to *E. coli* O86:B7. This strain was reported to express α-Gal detected by BSI-B_4 in multiple studies (18, 19, 45), and is frequently used as a positive control as it has also been shown to induce anti-α-Gal antibodies in *Ggta1* KO mice after oral inoculation (22). Surprisingly, neither 27H8 nor M86 antibody bound to *E. coli* O86:B7 while BSI-B_4 strongly stained this bacterial strain (Figure 5A). This was specific for *E. coli* O86:B7 because the negative control *E. coli* BL21, described in (46), was not stained by the lectin. We also tested further bacteria such as an *E. coli* strain isolated from human feces (*E. coli* HS) and *Lactobacillus rhamnosus* which showed minimal α-Gal positive staining by BSI-B_4 in (19). We could not observe any specific binding of 27H8 or M86 to these two strains (Figure 5A). BSI-B_4 did not bind to *E. coli* HS and showed a slight signal shift compared to the unstained control for *L. rhamnosus*. We could rule out technical errors of 27H8 applied in flow cytometry since α-Gal expressing HEK cells were indeed stained by this antibody using the same technique (Figure 2D). The binding of BSI-B_4 to *E. coli* O86:B7 in contrast to 27H8 and M86 could also be observed in a dot blot using lysates of this strain (Figure 5B). As it has been suggested that the induction of anti-α-Gal antibodies and also immunological tolerance towards this epitope might be driven by the intestinal microbiota (16), we wondered whether 27H8 binds to intestinal bacteria at all. Therefore, we incubated bacteria from the intestinal compartments of *Ggta1* KO mice with 27H8 for antibody binding and performed bacterial flow cytometry. To avoid anti-mouse secondary antibody attaching to murine immunoglobulins contained in the samples, we applied the biotinylated version of 27H8 and BSI-B_4 as control. While BSI-B_4-biotin bound to a large number of intestinal bacteria from the small intestine, cecum and colon, this was not visible for the biotinylated 27H8 antibody as there was no signal shift observable exceeding the streptavidin-PE (SAv-PE) only control or the biotinylated IgG1 control (Figure 5C). To confirm that also the biotinylated version of 27H8 binds to α-Gal in flow cytometry we applied the same technical setup as for the intestinal bacteria to splenocytes from *Ggta1* KO and WT mice. While biotinylated 27H8 bound to splenocytes from WT mice, no binding to splenocytes from *Ggta1* KO mice was detectable (Figure 5D). Altogether, we conclude that neither of the two α-Gal binding monoclonal antibodies 27H8 or M86 bind to structures on the bacterial surface or in lysates while the lectin BSI-B_4 indeed binds to bacterial epitopes most likely in a non-α-Gal epitope specific manner.

4. Discussion

Humans naturally display antibodies reactive to Gal-α1,3-Gal, the determining structure of the α-Gal epitope, that exhibit a broad range of pathogen reactivity and can also bind to non-α-Gal epitopes such as human blood group B, A and 0 (17). Different approaches have been used to purify such polyclonal and potentially cross-reactive anti-α-Gal antibodies (16, 47), yet they may also bind to non-Gal-α1,3-Gal expressing organisms (17). Previously, it has been hypothesized that anti-α-Gal antibodies are induced by the intestinal microbiota as *Escherichia coli*, *Klebsiella* and *Salmonella* strains can bind to polyclonal anti-α-Gal antibodies purified with Gal-α1,3-Gal-β1,4-Glc (16). For instance, oral inoculation of *Ggta1* KO mice with the *E. coli* strain O86:B7 has been shown to elicit enhanced anti-α-Gal titers (22). However, only the expression of α-Gal-like structures has been demonstrated for this strain to date as an additional fucose residue is attached to Gal-α1,3-Gal (48). To determine if bacteria express the α-Gal epitope defined as Gal-α1,3-Gal or Gal-α1,3-Gal-β1,4-GlcNAc without further residues attached to the second last galactose, the use of polyclonal, cross-reactive human anti-α-Gal antibodies might therefore lead to false positive results. Similarly, the lectins BSI-B_4 and MOA, binding also to α-Gal-like structures such as the blood group B antigen, do not exclusively recognize the α-Gal epitope. However, both polyclonal human anti-α-Gal antibodies and lectins have been used to demonstrate α-Gal epitope expression by bacteria and the microbiota in the past (18, 20).

Monoclonal antibodies allow a more precise epitope recognition after excluding cross-specificity as presented in this study. Here, we describe a novel, monoclonal IgG1 antibody called 27H8 which binds both the di- and trisaccharide α-Gal epitope with high affinity but does not display cross-reactivity to the blood group B antigen. Throughout the study, we demonstrate that the 27H8 monoclonal antibody binds to the same α-Gal containing structures as the most commonly used monoclonal IgM antibody called M86. We also show that M86 binds to both the di- and trisaccharide α-Gal epitope (Gal-α1,3-Gal or Gal-α1,3-Gal-β1,4-GlcNAc). The M86 antibody has been developed by Galili *et al.* in a similar approach by immunizing *Ggta1* KO mice with α-Gal rich rabbit red blood cells (24) in contrast to

synthetic α-Gal-OVA used in our study. Since SPR-affinity studies indicate that the K_Ds of the variable regions of the pentamer IgM antibody M86 genetically engineered to scFv-IgE antibodies (49) are higher than 27H8, we assume 27H8 variable regions bind to α-Gal at a higher affinity than M86. We used a broad screening approach utilizing cell lysates and purified α-Gal-rich proteins and lipids, and demonstrate specificity of 27H8 to α-Gal *via* enzymatic digestion, the use of *Ggta1* KO mice and pigs as well as transgenic expression of α1,3-galactosyltransferase in HEK cells. The 27H8 antibody recognizes α-Gal-conjugated proteins or natural α-Gal-rich compounds and glycolipids and is applicable in dot blot, immunohistochemistry, ELISA and flow cytometry, demonstrating robustness in its α-Gal epitope recognition. As the 27H8 antibody does not bind to the blood group B antigen, we conclude that further residues on the core galactose limits antibody binding to α-Gal. Additionally, this antibody displays unique features and advantages when compared to M86 as 27H8 is easily purifiable and can thus be directly labeled with fluorophores or enzymes for example to design improved ELISA systems.

As one first application, we used the 27H8 antibody to test the hypothesis if intestinal bacteria are a major source of α-Gal possibly involved in the sensitization of the immune system. Therefore, we applied the 27H8 antibody to lysates of bacteria hypothesized to be α-Gal expressing organisms *via* their expression of α1,3-galactosyltransferase-like genes (20). Strikingly, we did not detect any binding of 27H8 to *H. pylori*, *H. influenzae*, *S. typhimurium*, *P. aeruginosa, A. baumannii* and *A. muciniphila*. This lack of binding could also be demonstrated for *E. coli* O86:B7, another human *E. coli* isolate, *L. rhamnosus* and more generally for the majority of murine intestinal bacteria isolated from *Ggta1* KO mice. As this is a negative result, we cannot exclude that the 27H8 antibody binds to bacteria not tested in this setup or under different experimental conditions. However, as 27H8 also failed to stain murine intestinal bacteria derived from a host devoid of α-Gal, we propose that intestinal bacteria are generally devoid of the native α-Gal epitope. Similarly, the use of M86 for bacterial α-Gal epitope detection has not been shown convincingly and many studies relied on the use of lectins for this purpose (18–20). In our setting, we could equally not observe any binding of M86 when applied by flow cytometry or to lysates of cultured bacteria. We therefore conclude that either Gal-α1,3-Gal is not present on the tested bacteria and the intestinal microbiome, or it must be part of a more complex structure that shields antibody recognition by high-affine 27H8 and also M86. Consequently, the defining α-Gal epitope structure Gal-α1,3-Gal without further residues attached may not be expressed by bacteria at all. Another possibility might be that the α-Gal epitope is only revealed after processing the bacterial oligosaccharide structures by the host. Therefore, we strongly recommend to carefully differentiate between the expression of the actual α-Gal epitope, namely Gal-α1,3-Gal, and the expression of α-Gal-like glycans, e.g., α-Galactose residues connected *via* 1,3 linkages to other saccharides or further residues connected to the core galactose to avoid incorrect assumptions. In contrast to non-primate mammals and certain parasites, intestinal bacteria have been shown to express only α-Gal-like oligosaccharide structures (50) that may elicit initially low affine anti-α-Gal IgM antibodies. According to this scenario, a second yet to be discovered genuine α-Gal epitope source then triggers affinity maturation and IgG antibody production from this pool of B cells.

Additional methods to elucidate glycan structures on microbes may be nucleic magnetic resonance spectroscopy or reversed immunoglycomics as shown for *Leishmania major* (51). Moreover, we further encourage to carefully control experiments related to the destruction of the α-Gal epitope by using enzymatic digestion or periodate, as also described for anti-α-Gal IgE antibodies (39), to show the specificity of anti-α-Gal epitope recognition. We realized a binding of 27H8 antibody to *S. aureus* strains independent from the α-Gal epitope, an observation that was also made for human anti-α-Gal antibodies in another study. However, also in that study, these antibodies might have bound to protein A and not to the genuine α-Gal epitope (17).

In future studies, it will be interesting to apply 27H8 antibody to parasites suggested to express the actual α-Gal epitope, such as *Trypanosoma brucei* (52), *Ascaris lumbricoides* (53) and also *Plasmodium* species (18, 54) in order to investigate whether recognition of the α-Gal epitope is generally used by the immune system to recognize parasites. Additionally, 27H8 can be used to gain mechanistic insight into the 'red meat allergy' phenomenon mediated *via* tick bites (55), as compartmentalized α-Gal expression in tick species has been shown by an overlay staining of MOA and M86 (56). Lastly, xenotransplantation approaches of mammalian and in particular pig organs transplanted into human recipients heavily rely on the complete absence of the α-Gal epitope or the need to eradicate transplant reactive anti-α-Gal antibodies in the recipient prior to transplantation. The 27H8 antibody may be used to develop diagnostic tests and tools for α-Gal expression in diets and prior to organ transplantation and develop more sensitive sandwich ELISA tests to determine anti-α-Gal isotype levels in patients.

Altogether, we describe here a rigorously characterized and novel monoclonal IgG1 antibody that reliably recognizes the α-Gal epitope with high affinity and specificity. Using this novel tool, we propose to carefully re-evaluate bacterial α-Gal expression as a major epitope source and advocate for essential control stainings using several isotypes and enzymatic cleavage of the epitope to prove genuine α-Gal epitope expression in a given sample.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Ethics statement

The studies involving human participants were reviewed and approved by Ethical committee from the Technical University of Munich, School of Medicine, ethical vote numbers 419/18 S-KK and 5590/12. The patients/participants provided their written informed consent to participate in this study.

Author contributions

LK and CO designed the study. LK performed and analyzed most experiments. AS, RF, AF performed immunization protocols and monoclonal antibody generation. RJ performed affinity measurements. KS helped with flow cytometric analyses of HEK cells and bacteria. KF, MD, NC contributed to screening experiments. MK, LD, MSD contributed to bacterial experiments. MH, SE, DN, CS-W, SB, CH, TB contributed to experimental work or gave critical input. LK and CO wrote the manuscript with input from coauthors. All authors contributed to the article and approved the submitted version.

Funding

This work was supported by intramural funds from Helmholtz Munich and from the European Research Council (ERC Starting grant) project number 716718 to CO. The Ohnmacht lab is further supported by grants from the Deutsche Forschungsgemeinschaft (DFG) grant number OH 282/1-2 within FOR2599 to CO and project P07 within SFB1371 to CO. The Biedermann lab is supported by the DFG funded grants Project BI 969/12-1, the CRC1371 (Project P06), the CRC1335 (Project P17) and the RTG 2668. NC and CH were supported by a bilateral grant from the Luxembourg National Research Fund (FNR), project C17/BM/11656090 and the DFG.

Acknowledgments

We thank Johanna Grosch, Sabrina Engel and Alessandro De Sciscio for technical assistance, Maximilian Schiener for providing screening samples, Ann-Marie Maier for blood donor acquisition and, including Anna-Lena Geiselhöringer, Amelie Köhler, Maria Szente-Pasztoi, Daphne Kolland, Christian Hoffmann, Jinlong Ru and Florian Wölbing for helpful discussions.

Conflict of interest

MSD works as a consultant and an advisory board member at Theralution GmbH, Germany.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.958952/full#supplementary-material

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I opened myself to the gentle indifference of the world finding it so much like myself.

- Albert Camus