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

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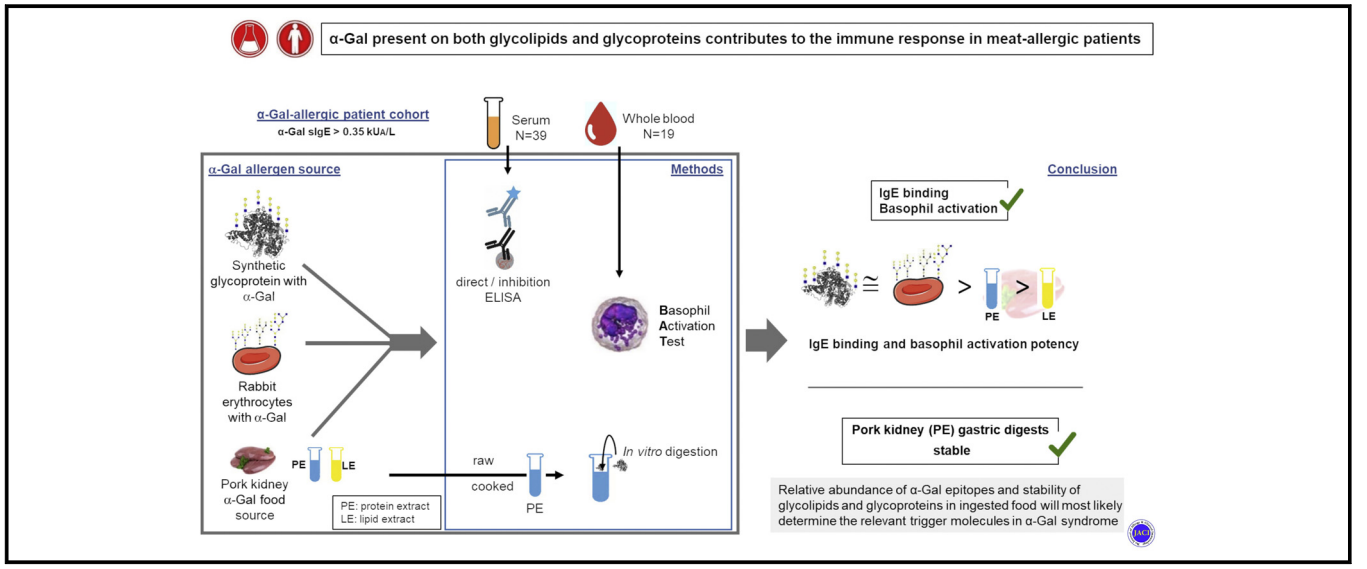
GRAPHICAL ABSTRACT



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

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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.

Available online April 19, 2022.

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0091-6749

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

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. 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;150:396-405.)

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

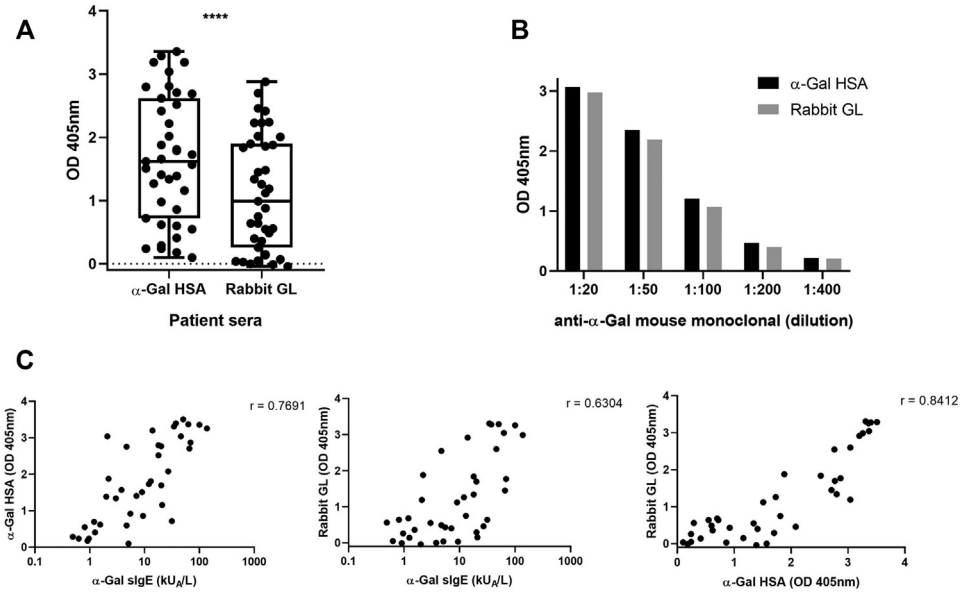

FIG 1. Glycolipids from rabbit erythrocytes recognized by IgE from α-Gal–allergic patient sera and by an anti–α-Gal antibody. (**A**) IgE reactivity of patient sera (n = 39; sIgE = 0.49-100 kU_A/L) against rabbit glycolipids (GL) and α-Gal HSA determined in ELISA. Box plot of optical density (OD) values 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 ($r = 0.8412$).

be present in a relatively large quantity (50% of the total glycans 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*).

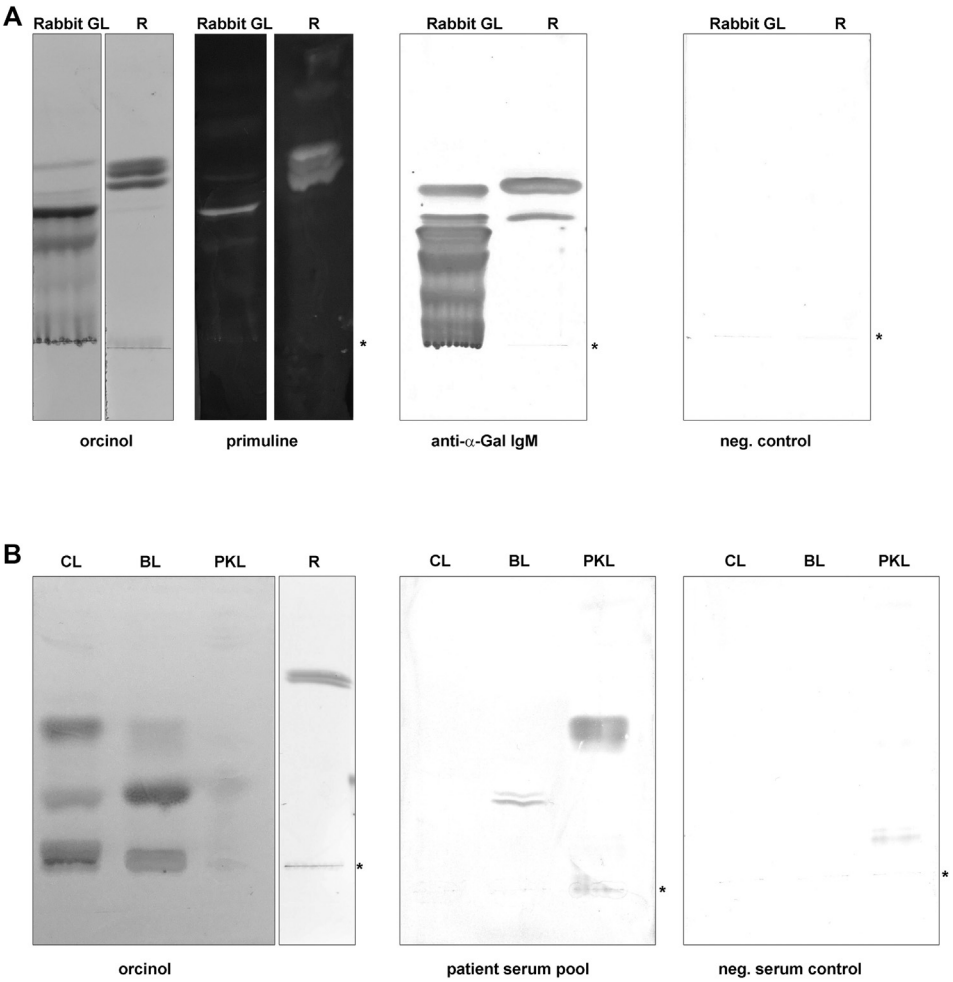

FIG 2. α-Gal on glycolipids recognized by anti–α-Gal antibodies. (**A**) TLC showing carbohydrates (orcinol staining) and lipids (primulin staining) in rabbit erythrocyte extract and reference glycolipids. TLC immunostaining of rabbit glycolipids with the murine monoclonal anti–α-Gal IgM (M86). R indicates 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

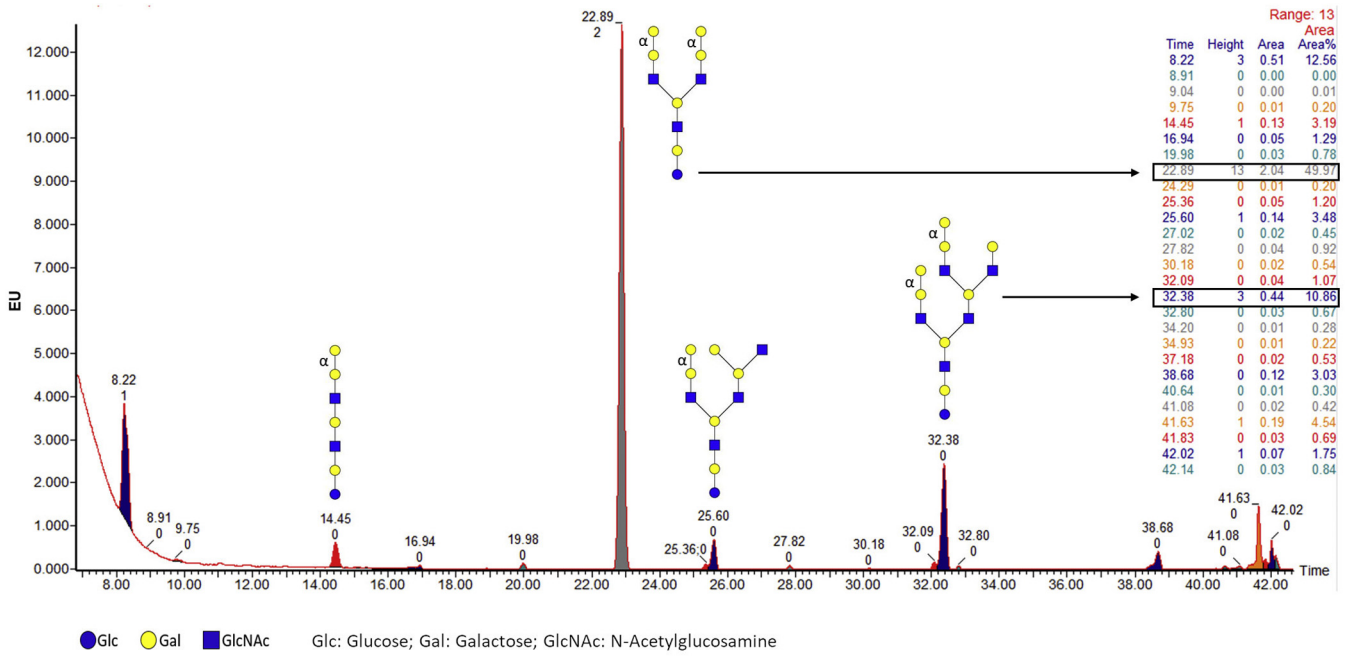

FIG 3. Numerous glycans with terminal α-Gal epitope, also referred to as rabbit glycolipids (*GL*), were detected in rabbit erythrocyte extract. Relative quantification of α-Gal glycolipids via ultra performance liquid chromatography coupled to fluorescence detector. Glycan nomenclature as per SNFG (Symbol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

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).

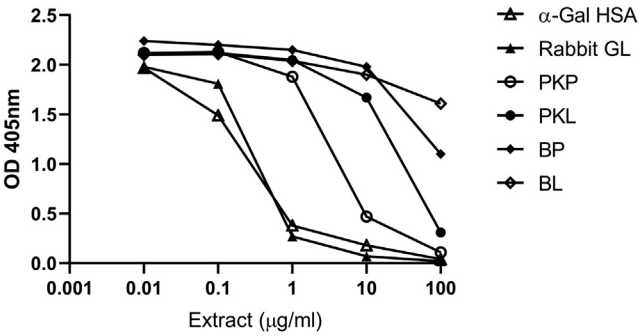

FIG 4. α-Gal–carrying molecules are present in a higher quantity in PK (**A**) and beef (**B**) protein extract than lipid extract. For inhibition ELISA, α-Gal HSA (2 μg/mL) precoated to a microtiter plate was detected by patient IgE (serum pool, sIgE = 74.8 kU_A/L; diluted 1:20) preinhibited with PK (*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).

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

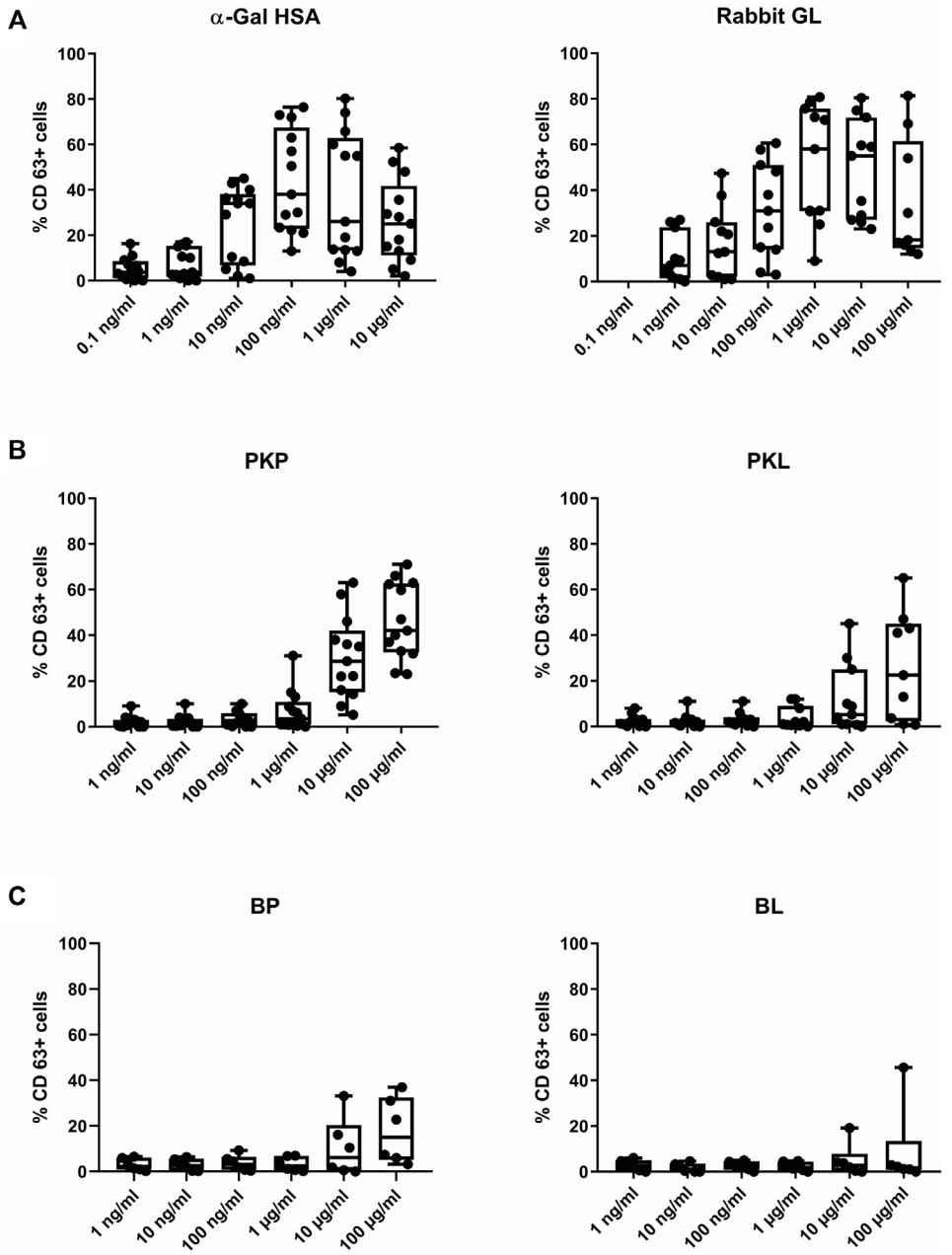

FIG 5. Basophils from clinically reactive α-Gal–allergic patients (sIgE 0.74-50.8 kU_A/L) are activated on stimulation with both α-Gal–carrying glycoproteins and glycolipids. Box plot with whiskers (minimum to maximum) showing percentage of CD63 upregulation with increasing dose of extracts in BAT assay. (**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

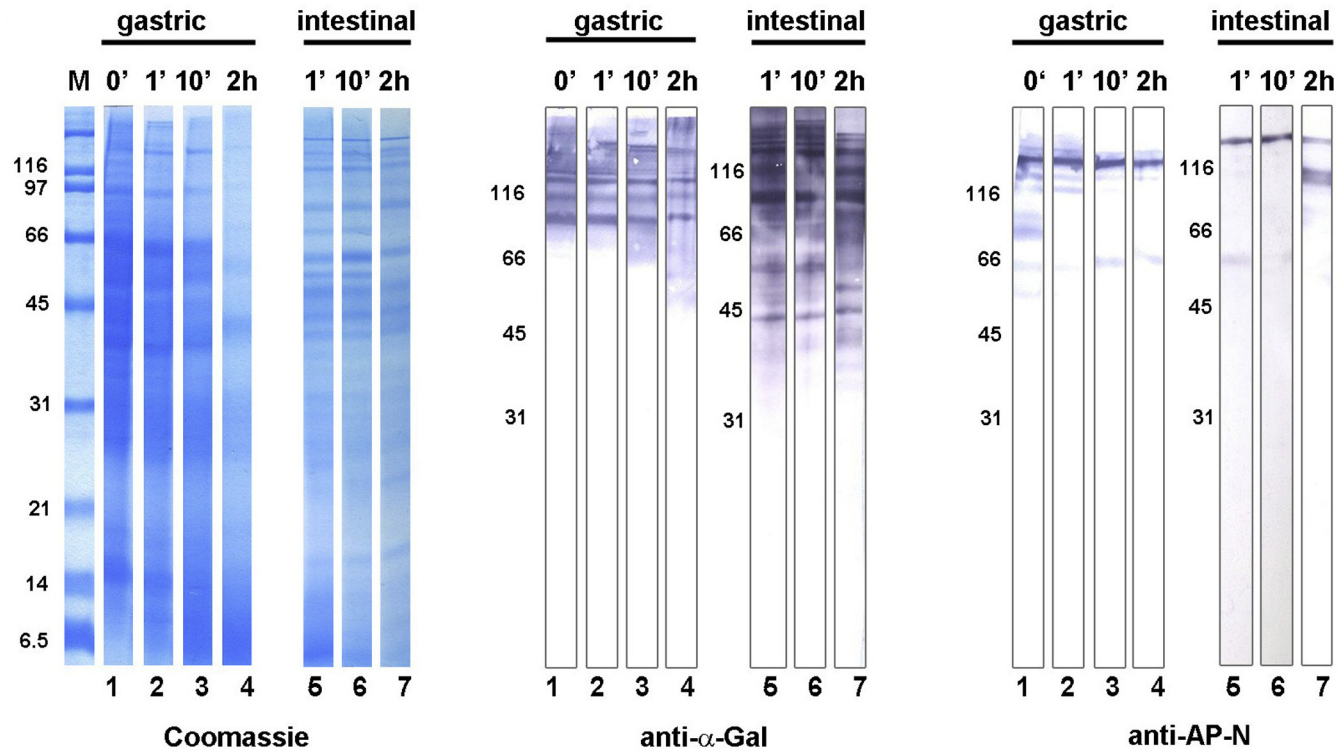

FIG 6. α-Gal–carrying glycoproteins in PK are highly stable under gastric digestion conditions. PK-soluble protein extract–Coomassie gel and immunoblot of simulated gastric and intestinal digest over a period of 0 minutes to 2 hours. Detection with anti–α-Gal and anti–AP-N antibodies in immunoblot. Time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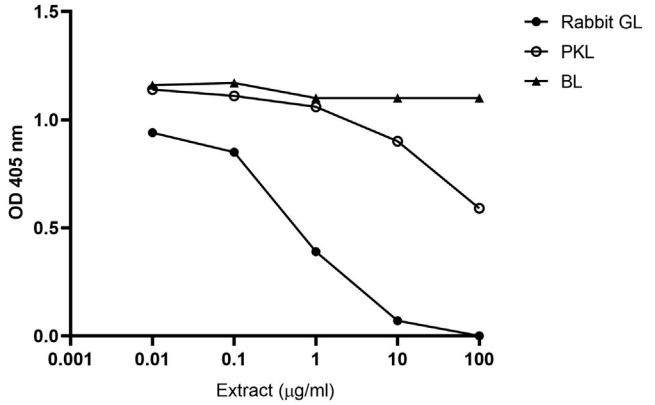

FIG E1. Detection of α-Gal–carrying glycans in lipid extracts with anti–α-Gal mouse monoclonal antibody M86. For inhibition ELISA, α-Gal-HSA (2 μg/mL) precoated onto a microtiter plate was detected via M86, which was previously inhibited with extracts (*BL,* beef lipids; *GL,* rabbit glycolipids; *PKL,*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

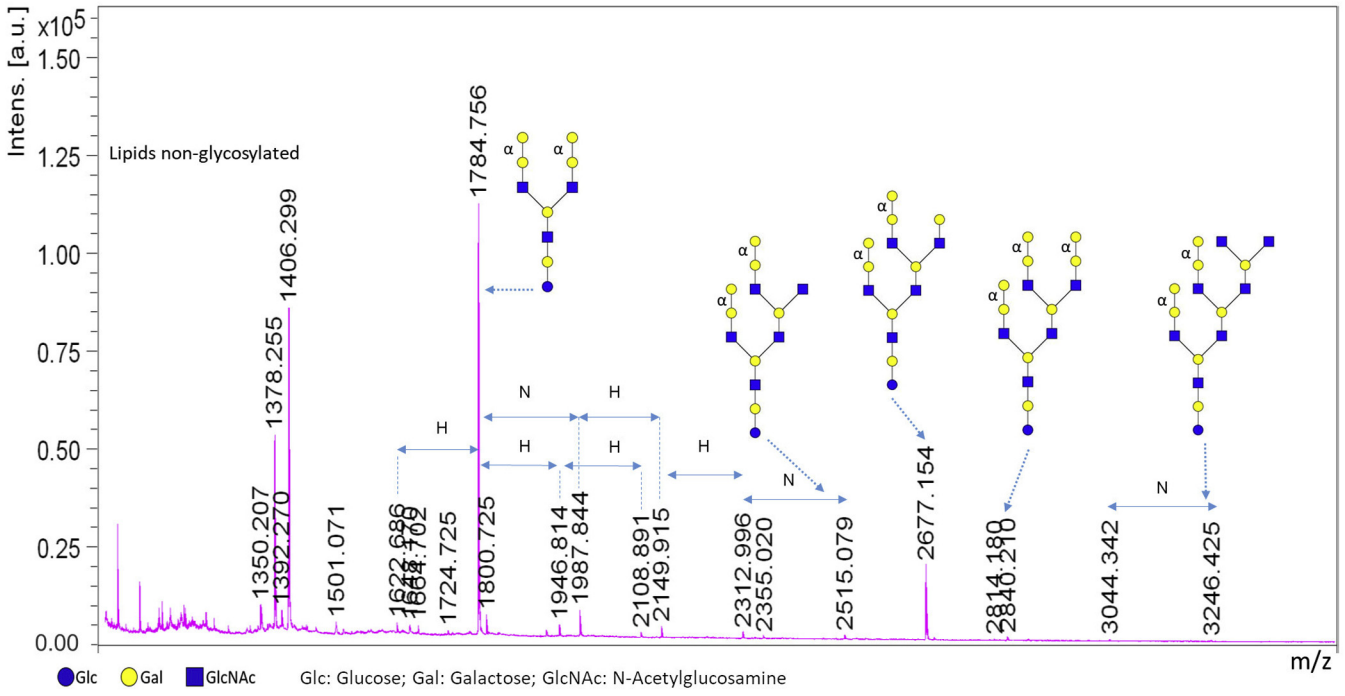

FIG E2. Numerous glycans with terminal α-Gal epitope detected in rabbit erythrocyte extract (rabbit glycolipids) via MALDI-TOF MS–based structure determination of glycans and glycomic analysis.

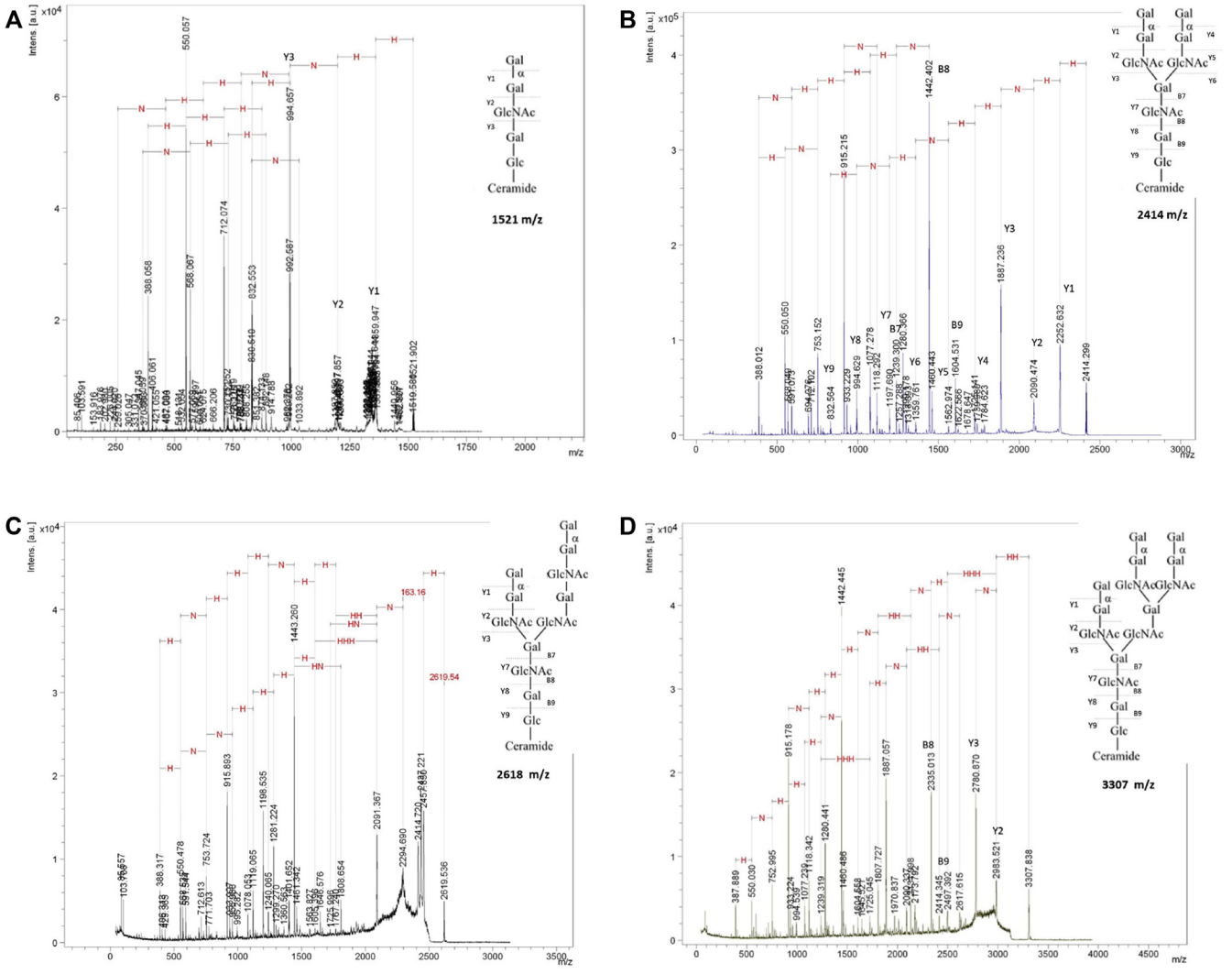

FIG E3. MALDI-TOF MS/MS analysis of rabbit glycolipids. Spectra show α-1,3 linkage determination in glycans from 4 α-Gal–carrying rabbit glycolipids.

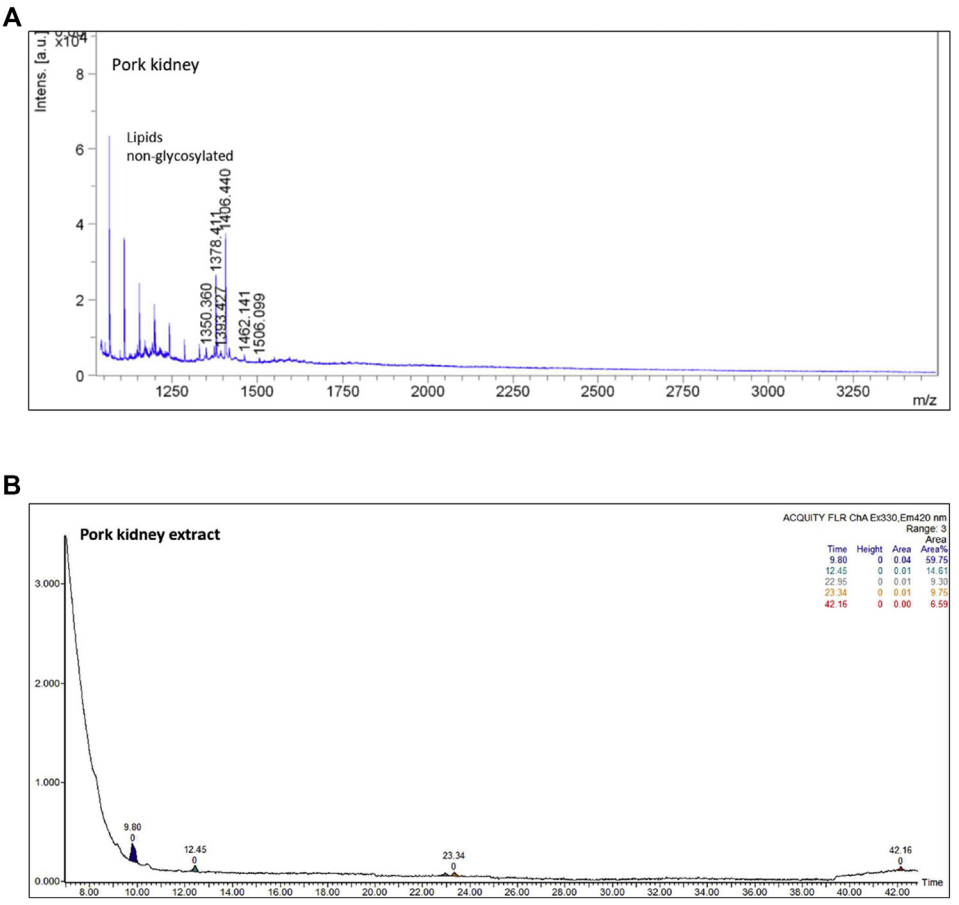

FIG E4. Glycomic analysis of PK lipid extract failed to detect α-Gal–carrying glycans as a result of their low abundance. (**A**) MALDI-TOF MS–based identification of glycans; *x-axis* indicates m/z; *y-axis,* intensity (AU, arbitrary units) $\times 10^4$. (**B**) Relative quantification of glycolipids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

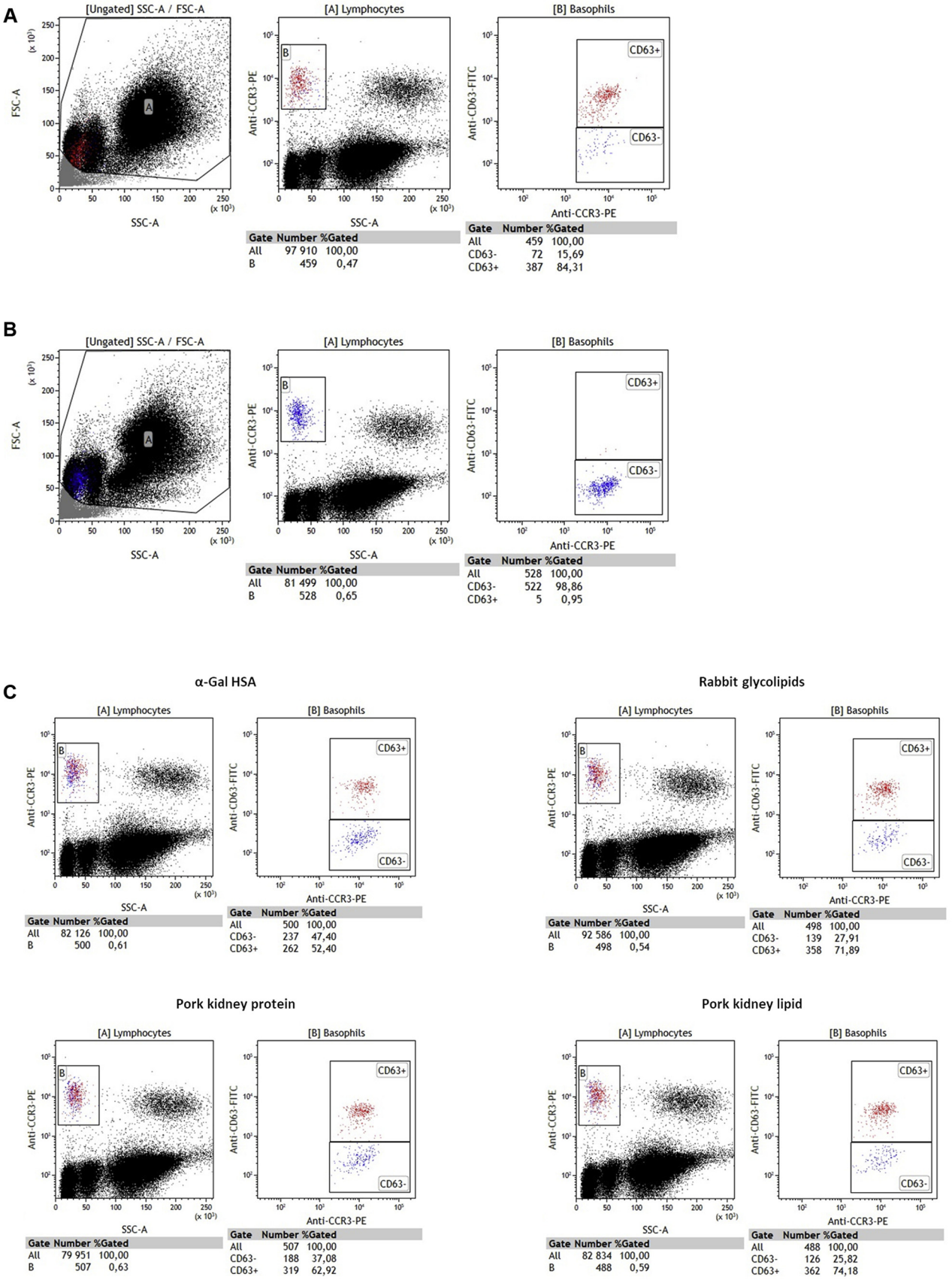

FIG E5. Basophil gating strategy and activation plots. (**A**) Positive control (anti-FCεRI). (**B**) Negative control (unstimulated). (**C**) Scattered dot plots showing upregulation of CD63 obtained after stimulation with α-Gal HSA (10 μg/mL; *top left*), rabbit glycolipids (10 μg/mL; *top right*), 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.

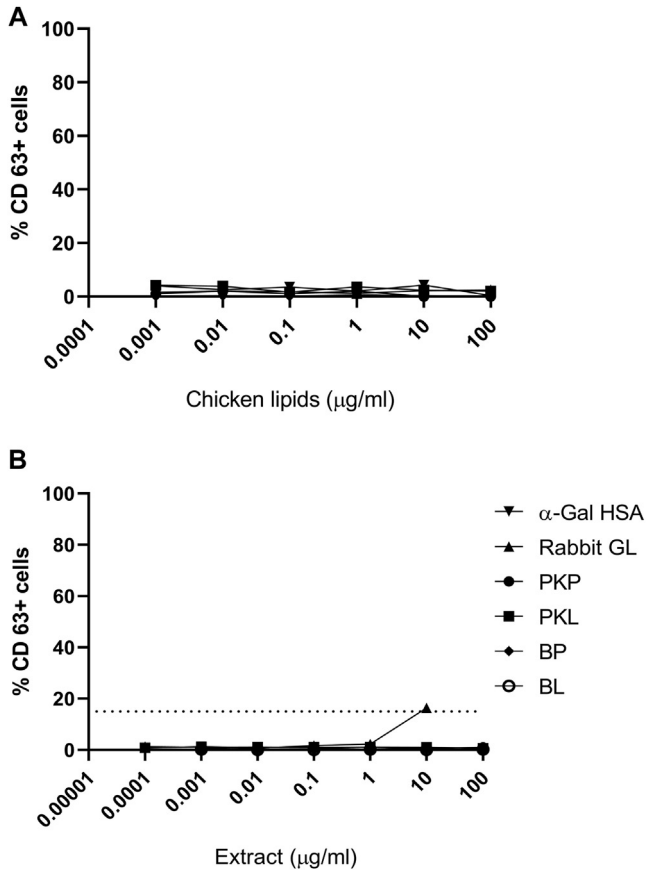

FIG E6. (**A**) Basophils from clinically reactive α-Gal–allergic patients (n = 5; sIgE 0.91-50.8 kU_A/L) are not activated on incubation with chicken lipids at varying concentrations. Percentage of CD63-positive cells (*y-axis*) vs chicken lipid extract (*x-axis*). (**B**) Basophils of allergic or healthy 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%).

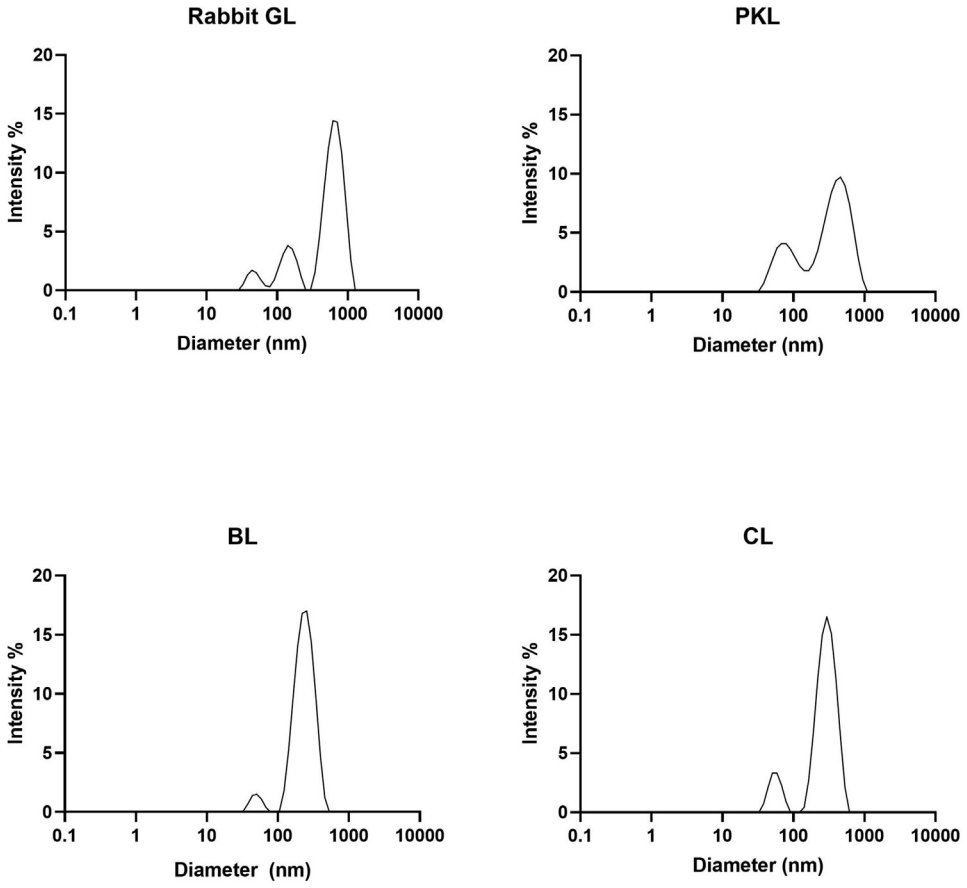

FIG E7. Glycolipids form micelles when dissolved in a polar solvent. Micelles formed by rabbit glycolipids (*GL*) and PK lipid (*PKL*), beef lipid (*BL*), and chicken lipid (*CL*) extracts as observed by dynamic light scattering. *X-axis* indicates diameter of micelles (nm); *y-axis,* percentage intensity.

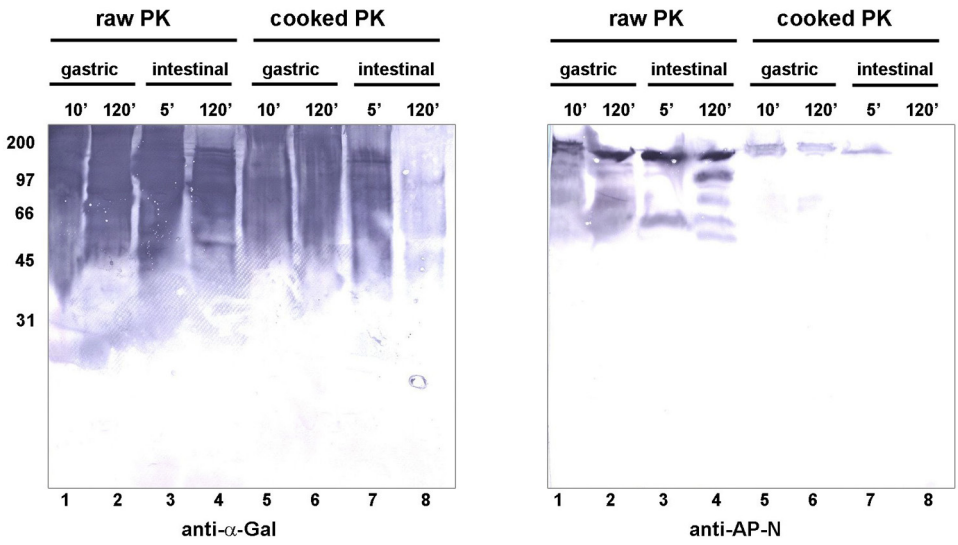

FIG E8. α-Gal–carrying glycoproteins in PK are stable under gastric digestion conditions. Complete PK extract (soluble and insoluble proteins) immunoblot of simulated gastric and intestinal digest from raw and cooked PK; detection with anti–α-Gal and anti–AP-N antibodies. Time point of sample analysis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