RESEARCH ARTICLE

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Milk Fat Globule Proteins Are Relevant Bovine Milk Allergens in Patients with α-Gal Syndrome

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Alpha-gal syndrome (AGS) is a mammalian meat allergy associated with tick bites and specific IgE to the oligosaccharide galactose-α-1,3-galactose (α-gal). Recent studies have shown that 10–20% of AGS patients also react to the dairy proteins. Considering the already described role of the meat lipid fraction in AGS manifestations, the aim of this work has been to investigate whether the milk fat globule proteins (MFGPs) could be involved in AGS. The MFGPs are extracted and their recognition by the IgE of AGS patients is proved through immunoblotting experiments. The identification of the immunoreactive proteins by LC-HRMS analysis allows to demonstrate for the first time that butyrophillin, lactadherin, and xanthine oxidase (XO) are α-gal glycosylated. The role of xanthine oxidase seems to be prevalent since it is highly recognized by both the anti-α-gal antibody and AGS patient sera. The results obtained in this study provide novel insights in the characterization of α-Gal carrying glycoproteins in bovine milk, supporting the possibility that milk, especially in its whole form, may give reactions in AGS patients. Although additional factors are probably associated with the clinical manifestations, the avoidance of milk and milk products should be considered in individuals with AGS showing symptoms related to milk consumption.

1. Introduction

Alpha-gal syndrome (AGS) is a mammalian meat allergy associated with tick bites and specific IgE antibodies to the oligosaccharide galactose-α-1,3-galactose (α-gal).[1,2] Alpha-gal carbohydrate is missing in humans and some primates, since the α-1,3-galactosyltransferase is expressed in an enzymatically inactive form. It is instead present in most mammals, many pathogens such as bacteria and parasites and in the salivary glands of several tick species, including the most prevalent hard tick in Europe (*Ixodes ricinus*).[3] Anti α-gal antibodies are the most abundant natural antibodies in humans and some primates constituting up to 1% of the circulating antibodies. These antibodies are mainly IgM and IgG class, but anti α-Gal IgEs can be also produced in some individuals suffering from the red meat allergy.[4] AGS symptoms vary from abdominal pain and diarrhea to urticaria and anaphylaxis, the latter being experienced by nearly 50% of patients.[5,6] AGS shows several exclusive features that make it different from other food allergies: i) reactions are generally delayed, appearing 3–6 h after meat consumption; ii) IgE antibodies react to a carbohydrate moiety rather than a protein epitope; iii) patients can develop AGS in late adulthood after a previous period of meat tolerance.[7] This atypical food allergy was first described in the southeastern regions of the United States and in Australia, but it was also reported soon thereafter in Europe, Asia, Africa, and Central America.[8] More than 450 000 cases have been described to date in the United States.[9] The frequency of positivity of specific IgE to α-Gal in Europe has been reported to be increasing in

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northern countries (Denmark, Sweden, etc.), where it was first investigated,[10,11] but also in Spain[12] and in the rural areas of northeast Italy.[13] AGS is characterized by reactions to mammalian meat and innards, including beef, pork, and lamb, as well as to food gelatins and some medications (cetuximab, antivenom, and gelatin-containing vaccines).[14] Unlike common food allergies, the allergic reactions may not occur at every exposure to the allergen. This variability depends on the amount of allergen ingested and on the nature of the biologic macromolecules within the α-gal-containing food. Lipid-rich mammalian meats are associated with more consistent and severe reactions.[15] Lipid-bound α-Gal appears to be able to cross the intestinal monolayer and to trigger an allergic reaction, thus suggesting that not only glycoproteins but also glycolipids should be investigated as potential allergenic molecules.[16] Chakrapani et al.[17] have recently confirmed the involvement of glycolipids in the activation of AGS patient basophils, even if the major role played by glycoproteins, particularly those from pork kidneys and beef extracts, is already well established. Glycolipids extracted from these food matrices have shown a lower basophil activation capacity than their respective protein extracts.[18]

Not only red meat but also bovine milk contains α-Gal-epitopes, although in smaller amounts.[19] Some recent studies,[7,20,21] including one considering a large cohort of 2500 AGS patients in the USA,[22] have demonstrated that 10–20% of AGS patients also react to milk. The most reported symptoms in AGS patients following bovine milk ingestion are abdominal pain and urticaria with a delayed onset of the symptoms.[23] Unlike meat, where α-Gal-bearing proteins have long been extensively studied, sources containing α-Gal epitopes in dairy products have only recently been investigated,[24] by a modified inhibition RIA (Radioimmunoassay), were able to detect α-Gal proteins in heavy milk cream but no detectable α-Gal was found in skimmed cow milk or 1%, 2% milk fat. Perusko et al.[25] demonstrated that bovine γ-globulin (BGG), lactoferrin (LF), and lactoperoxidase (LPO) are α-Gal carrying proteins that have been recognized by the IgEs of AGS patients and which are able to activate the basophils of patients. More recently, the same α-Gal glycosylated proteins were found in sheep milk by German-Sanchez et al.[26]

Milk lipid fraction consists of lipid globules surrounded by membrane. The MFG is assembled and secreted by the epithelial cells of the mammary gland and consists of a complex mixture of proteins, enzymes, neutral lipids, and phospholipids enriched with glycoproteins leaving the mammary cell by exocytosis.[27] During the last years MFG proteins (MFGP) have been reported to have impact on several cellular processes such as inflammation, differentiation, antimicrobial and antiadhesive properties, and proliferation of intestinal epithelial cells.[28] MFGP represent 1–4% of the total milk proteins. The major MFGP in bovine milk are adipophilin (ADPH), butyrophilin (BTN), mucins (MUC1, MUC 4, and MUC15), xanthine dehydrogenase/oxidase (XDH/XO), CD36, lactadherin (LA), periodic acid Schiff III (PAS III), and fatty acid-binding protein (FABP).[29] Proteomics has been employed in the identification and characterization of MFGP.[30,31] In term of glycoproteomic studies, MFGM *N*-glycoproteins, including butyrophilin, lactadherin, mucins, integrins, and immunoglobulins, have been successfully studied.[28,32] Less is known concerning the galactose-α-1,3-galactose determinant on bovine MFGP.

Considering the involvement of milk proteins in AGS, the proposed role of the lipid fraction in facilitating clinical manifestations of AGS, and the recent considerations reported by Wilson et al.[33] indicating high-fat dairy products as more problematic than light milk, the aim of this work is to investigate whether the milk fat globule protein fraction may play a role in AGS.

Table 1. Patients enrolled in the study.

Patient ID	Sex	Age	Culprit food	Alpha gal IgEs [KUA L^{-1}]
Alpha1	M	37	Since 2020: red meat	3.08
Alpha2	F	74	2017: veal kidney 2018: tripe	0.37
Alpha3	F	69	2017: boiled meat and soup with beef broth 2018: offal 2019: veal broth.	86.50
Alpha4	M	68	Since 2018: offal 2022: stuffed meat	15.01
Alpha5	F	74	2017: lamb stew 2018: lamb liver and lung	2.54
Alpha6	M	66	2018: "capocollo" 3 more similar episodes after ingestion of pork or offal	11.60
Alpha7	M	57	2010-2014: gummy bears 2016: red meat 2017: rabbit liver	>100
Alpha8	M	58	2018: meat	31.50
Alpha9	F	26	Since 2015: red meat	1.17
Alpha10	M	67	Since 2010: meat	8.95
negative control	M	48		<0.10

F: female; M: male.

2. Results

2.1. Study Population

Ten adult patients (4 females; 40.0%) with a mean age of 59.4 years (range 25–74 years) and a diagnosis of α-gal syndrome (AGS) were enrolled in the experiment. One patient (M, 48 years) not consuming meat and without any history of food allergies was used as negative control.

2.1.1. Clinical Presentation of AGS

All the AGS patients reported at least one delayed reaction (average 3.40 ± 1.58 events per person) with a mean onset time of 4.1 h after eating red meat, innards, or meat-related food (**Table 1** and Table S1, Supporting Information). None of the patients were allergic to cow's milk. The most common culprit food was pork meat. Urticaria was the most common clinical manifestation (100%), followed by gastrointestinal symptoms

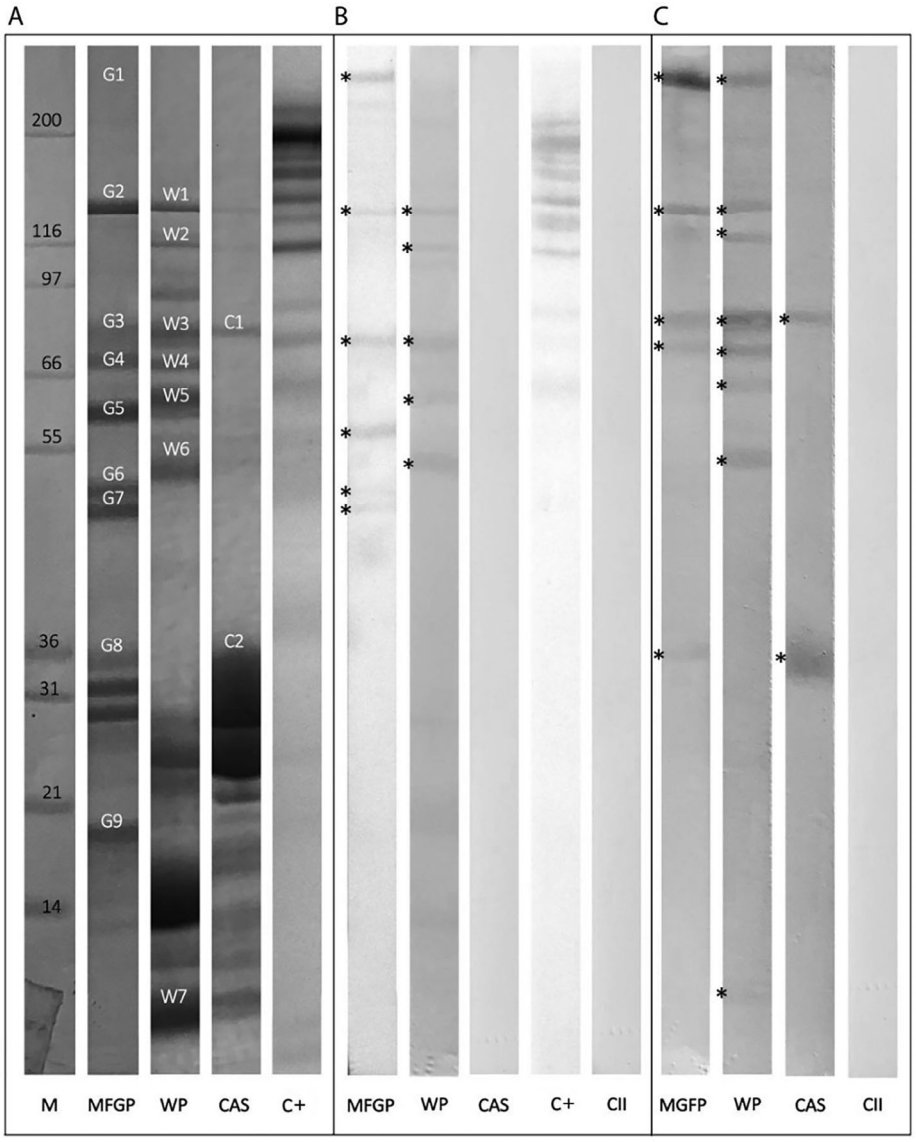

Figure 1. Investigation of the three bovine milk fractions: caseins (CAS), whey proteins (WP) and milk fat globule associated proteins (MFGP). A) LDS page of MFGP, WP and CAS. B) Immunoblotting of MFGP, WP, and CAS with the anti-α-Gal IgG antibody. C) Immunoblotting of MFGP, WP, and CAS with the sera of a pool of 10 AGS patients. M: molecular weight markers; C+: thyroglobulin; CII: secondary antibody control.

(vomiting, diarrhea, and abdominal pain) (60%), hypotension (50%), angioedema (50%), and dyspnea (30%). Nine patients (90%) had at least one episode of anaphylaxis, diagnosed according to NIAID/FAAN criteria.[34] No cofactor of anaphylaxis, including ethanol, or nonsteroidal anti-inflammatory drug consumption was identified, apart from one patient who reported anaphylaxis after red meat ingestion and physical exercise. None of our patients had previously been treated with cetuximab. Eight patients (80%) reported one or multiple tick bites before AGS.

All the patients were positive to α-gal specific IgE (26.08 $\pm$ 35.87 KUA L^{-1}) with a mean serum total IgE of 389.99 $\pm$ 429.94 KU L^{-1}. Tryptase resulted normal in all the patients, with a mean value of 7.18 $\pm$ 3.78 µg L^{-1}).

All the patients received corticosteroids and antihistamines for their hypersensitivity reactions. Seven patients (70%) had been admitted to the intensive care unit for a total of 10 times. In five cases, the reactions were treated with adrenaline.

2.2. The Anti-α-Gal Antibody Recognizes Whey and Milk Fat Globule Proteins

The milk fat globule proteins (MFGP), whey proteins (WP), and caseins (CAS) were separated by means of LDS page followed by immunoblotting analysis with anti-α-Gal IgG and a pool of sera from 10 AGS patients (**Figure 1**). Both the MFGP and WP extracts showed immunoreactive bands for anti-α-Gal IgG: G1, G2, G3, G5, G6, G7 and W1, W2, W3, W5, W6, respectively (Figure 1 B). LC-HRMS analysis (**Table 2** and Table S2, Supporting

Table 2. Identification of the proteins immunorecognized by anti-α-Gal IgG and/or by the pool of α-Gal syndrome patient's sera in the milk fat globule membrane protein (band from G1 to G25), whey protein (from W1 to W7) and casein fractions (C1 and C2).

No Band	Entry	Protein name	MW_{EXP}/MW_{THEOR} [DA]	Protein score > 35	No of matching peptides (>3)
G1	P80457	Xanthine oxidase	300 000/142 330	130.48	16
	Q8WNR8	Perilipin	300 000/45 251	52.718	8
	Q27960	Sodium-dependent phosphate transport protein 2B	300 000/75 825	52.365	5
	Q4GZT4	ATP-binding cassette transporter ABCG2	300 000/72 724	43.296	7
G2	P80457	Xanthine oxidase	130 000/146 790	317.14	30
	P18892	Butyrophilin subfamily 1 member A1	130 000/59 231	66.673	11
G3	G5E5T5	Ig-like domain-containing protein	80 000/55 968	129.03	10
	A0A3Q1M193	Glycoprotein 2	80 000/58 465	260.53	8
	P18892	Butyrophilin subfamily 1 member A1	80 000/59 276	145.46	10
	C7FE01	Lactoferrin	80 000/80 278	55.906	8
G4	P81265	Polymeric immunoglobulin receptor	68 000/82 434	211.62	18
	A0A3Q1M193	Glycoprotein 2	68 000/58 465	92.215	10
	P18892	Butyrophilin subfamily 1 member A1	68 000/59 276	106.11	15
	P26201	Glycoprotein IIIb	68 000/46 055	91.212	6
	G5E513	Ig-like domain-containing protein	68 000/48 107	95.157	9
	A0A3Q1LWT4	Acyl-CoA synthetase long chain family member 1	68 000/81 442	79.564	10
	J7K1V4	Lactoferrin	68 000/80 278	75.774	12
	F1MHI1	Perilipin	68 000/45 281	53.926	7
	A0A3Q1MK38	Terpene cyclase/mutase family member	68 000/74 156	52.104	5
G5	P18892	Butyrophilin subfamily 1 member A1	60 000/59 276	252.71	19
	Q95114	Milk fat globule-EGF factor 8 protein (Lactadherin)	60 000/43 140	50.477	6
G6	Q95114	Milk fat globule-EGF factor 8 protein (Lactadherin)	51 000/43 140	198.57	22
	Q9TUM6	Perilipin-2	51 000/49 368	189.24	19
G7	Q95114	Milk fat globule-EGF factor 8 protein (Lactadherin)	49 000/43 140	231.35	13
	Q8HZM7	Perilipin	49 000/45 281	55.801	4
G8	P02663	Alpha-S2-casein	34 000/26 018	41.439	6
	P18892	Butyrophilin subfamily 1 member A1	34 000/59 231	47.768	5
G9	B5B0D4	Major allergen beta-lactoglobulin	19 000/19 969	116.59	11
	Q5E9I6	ADP-ribosylation factor 3	19 000/20 601	47.494	7
G10	P80457	Xanthine oxidase	300 000/142 330	37.778	5
G11	P80457	Xanthine oxidase	170 000/142 330	97.052	11
G12	P80457	Xanthine oxidase	130 000/146 790	167.66	20
G13	P80457	Xanthine oxidase	116 000/14 233	103.51	11
G14	G5E5T5	Immunoglobulin heavy constant mu	80 000/56 043	157.78	12
	F1MZQ4	Butyrophilin subfamily 1 member A1	80 000/59 231	65.44	7
G15	A0A4W2DWX4	Butyrophilin subfamily 1 member A1	60 000/59 245	94.962	13
G16	P0DOX5	Immunoglobulin gamma-1 heavy chain	53 000/49 328	97.277	10
G17	P01834	Immunoglobulin kappa constant	28 000/11 765	59.743	5
G18	P80457	Xanthine oxidase	300 000/146 690	17.852	3
G19	P80457	Xanthine oxidase	130 000/146 790	292.24	32
G20	G5ES13	Immunoglobulin heavy constant mu	60 000/56 043	84.106	10
	P81265	Polymeric immunoglobulin receptor	60 000/82 434	65.441	9
	P18892	Butyrophilin subfamily 1 member A1	60 000/59 276	51.22	8
G21	F1MZQ4	Butyrophilin subfamily 1 member A1	57 000/59 231	63.366	7
G22	P18892	Butyrophilin subfamily 1 member A1	55 000/59 231	143.59	16
G23	Q9TUM6	Perilipin-2	48 000/49 368	83.058	8
	P18892	Butyrophilin subfamily 1 member A1	48 000/59 276	45.368	6
G24	Q95114	Milk fat globule-EGF factor 8 protein (Lactadherin)	44 000/43 140	137.39	16

(*Continued*)

Table 2. (Continued)

No Band	Entry	Protein name	MW_{EXP}/MW_{THEOR} [DA]	Protein score > 35	No of matching peptides (>3)
G25	P21163.2	asparagine amidase PNGase F	40 000/39 032	227.36	16
	P18892	Butyrophilin subfamily 1 member A1	40 000/59 276	49.619	5
W1	P80457	Xanthine oxidase	130 000/146 790	323.31	20
W2	A0A4W2CZN6	C3 complement	110 000/190 950	308.81	32
	A0A3Q1M3L6	Ig-like domain-containing protein	110 000/40 475	106.25	7
W3	C7FE01	Lactoferrin	75 000/76 274	323.31	45
	G5E513	Ig-like domain-containing protein	75 000/48 107	307.5	16
	G3×6N3	Serotransferrin	75 000/77 738	117.08	22
	P80025	Lactoperoxidase	75 000/71 350	187.39	22
	A0A3Q1M3L6	Ig-like domain-containing protein	75 000/40 475	44.51	4
	B3VTM3	Lactotransferrin	75 000/78 056	45.075	7
W4	P81265	Polymeric immunoglobulin receptor	68 000/82 434	134.53	11
	A0A4W2DZ09	Serotransferrin	68 000/77 738	133.09	15
	E1BMJ0	Serpin family G member 1	68 000/51 772	95.139	5
	A0A4W2CZN6	C3-beta-c	68 000/190 950	60.754	10
	A0A3Q1M032	Ig-like domain-containing protein	68 000/40 475	79.946	4
	A0A4W2DDL5	Albumin	68 000/68 198	60.754	8
W5	P02769	Albumin	60 000/68 198	323.31	41
	A0A4W2CZN6	C3 complement	60 000/190 950	244.45	28
W6	A0A3Q1M3L6	Ig-like domain-containing protein	50 000/40 475	148.91	10
	G3N0V0	Ig-like domain-containing protein	50 000/35 951	49.249	6
	Q9TTE1	Serpin A3-1	50 000/46 236	75.075	7
W7	P00711	Alpha-lactalbumin	15 000/14 156	144.24	3
C1	P24627	Lactotransferrin	75 000/78 056	323.31	47
	P18892	Butyrophilin subfamily 1 member A1	75 000/59 276	41.743	3
C2	P02662	Alpha-S1-casein	27 000/23 689	323.31	8
	A0A140T8A9	Kappa-casein	27 000/21 237	190.77	4
	A0A452DHW7	Beta-casein	27 000/29 221	62.074	5
	P02754	Beta-lactoglobulin	27 000/19 883	61.784	5

Information) allowed LF and LPO to be identified in band W3, and several Ig-like domains containing proteins were identified in bands W2, W3, W5, and W6. Xanthine Oxidase (XO) was identified in W1 and in several reactive bands of MFGP (G1, G2, and G3), while the other reactive bands (G3, G5, G6, and G7) mainly contained butyrophilin (BT) and lactadherin (LA).

The pool of AGS patient sera immunorecognized all the bands already recognized by anti-α-Gal IgG, albeit with the addition of bands G4, G8, W4, W7, C1 and C2. Band G4 contained several proteins including XO, BT and LA; G8, W4 and C1 contained already known α-Gal glycosylated proteins (BT, LPO and Ig-like domain-containing proteins); while bands W7 and C2 contained typical milk allergens (β-lactoglobulin and caseins) and were probably recognized because the patients were sensitized to milk, although they tolerated it well, according to the study inclusion criteria (Figure 1C).

Band G1, which contained XO, was not visualized by colloidal Coomassie staining or even by silver staining (data not shown), but it was clearly recognized by anti-α-Gal IgG and by the AGS patient IgEs in the immunoblotting experiment.

2.3. Xanthine Oxidase, Butyrophilin, and Lactadherin Are α-Gal-Glycosylated Proteins

In order to enrich the sample in α-Gal-glycosylated proteins, we isolated glycosylated MFGP using BioMag Goat Anti-Human IgG beads conjugated with the anti-α-gal IgG system. After the enrichment, the proteins were separated by means of LDS PAGE (**Figure** 2A, lane MFGPb). The thus isolated MFGP resulted to be high molecular weight proteins and as expected, they were recognized by the anti-α-gal IgG. However, the situation was different for bands G16, G17, and G25, as they contained heavy and light anti-α-gal IgG chains partially released from the beads during protein elution, and PNGase F, the enzyme used for deglycosylation. In addition to the heavy anti-α-gal IgG chain, LA was identified in band G16, which is probably responsible for the corresponding immunoreactivity, while the other two bands did not result to be immunoreactive. When the α-Gal-enriched protein sample was de-glycosylated with PNGase F, the anti-α-gal IgG did not recognize any band, except for a slight recognition of G18 where XO was present (Figure 2A, lane MFGPb-DEG). This reactivity completely disappeared only after a more exhaustive overnight PNGase F de-glycosylation (Figure 2A, lane

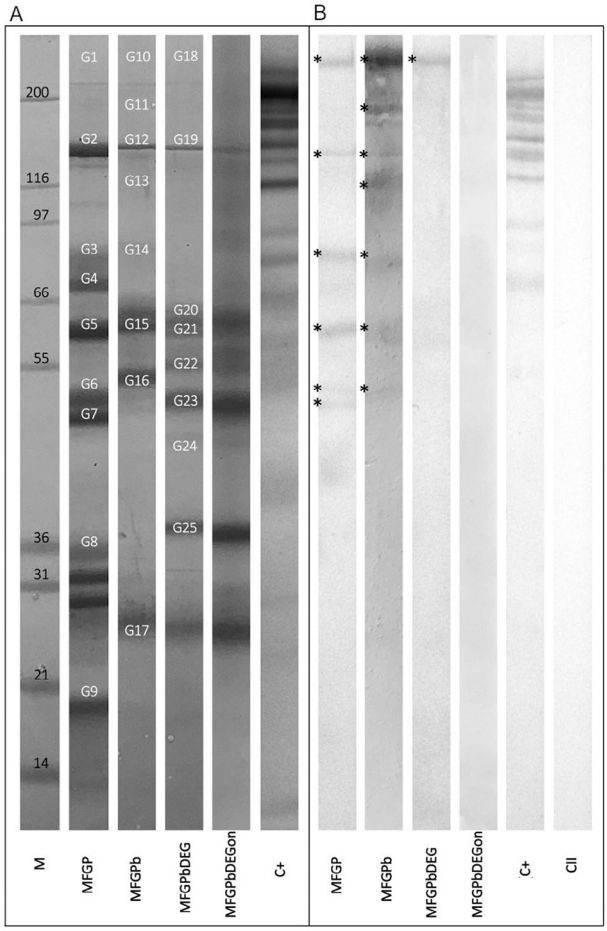

Figure 2. Investigation of α-gal bovine milk proteins. A) LDS page of milk fat globule associated proteins (MFGP). MFGP enriched by means of incubation with beads bound with anti-α-gal IgG (MFGPb) and MFGPb de-glycosilated with PNGase for 3 h (MFGPbDEG) and overnight (ON) (MFGPbDEGon). B) Immunoblotting of MFGP, MFGPb, MFGPbDEG, and MFGPbDEGon with anti-α-gal IgG. M: molecular weight; C+: thyroglobulin; CII: secondary antibody control.

MFGPbDEGon). The analysis of the bands containing the N-deglycosylated proteins that lost reactivity revealed which asparagine could carry the α-gal moiety (Figure 2A,B, lane MFGPbDEG). The presence of new tryptic peptides with aspartic acid instead of the original asparagine was considered as proof of the presence of a glycosylation site carrying the α-gal sugar chain on the peptide before digestion. The LC-HRMS study of the G22 band showed a BT peptide with Asn_{215} modified to Asp_{215} after the de-glycosylation protocol. The same was observed in band G24, where LA showed an Asn_{227} modified to Asp_{227}. All these results are summarized in **Table 3**.

2.4. The AGS Patients' IgE Antibodies Recognize Xanthine Oxidase and Butyrophilin

The MFGP sample was also incubated with the serum of each single patient (**Figure 3**). As in previous experiments, the most recognized bands were G1 (recognized by 7/10 patients), G2 (8/10 patients), and G4 (8/10 patients), which mainly contain XO and BP. Bands G5 and G8, which showed a reduced recognition rate, were recognized by 2/10 patients, while G6 was recognized by 3/10 patients and G9 by only 1 patient. Once again, these bands mainly contained XO, but also LA and β-lactoglobulin.

In order to verify that the patient immunorecognition was addressed to α-gal epitopes, immunoprecipitation of three patients' sera (α2, α3, and α5) was performed with four concentrations of bovine thyroglobulin (1, 3, 30, and 60 µg) (**Figure 4A**). Only patient α3 needed 60 µg of thyroglobulin to completely inhibit the immunorecognition. Instead, for the other two patients, 3 or 30 µg was sufficient. The same experiment was performed with bovine XO (patients α1, α2, and α7) (Figure 4B). In this case, 60 µg of XO was needed to immunoprecipitate the patients' sera. Patient α2, who was tested in both inhibition experiments, needed 60 µg of XO and only 3 µg of thyroglobulin.

3. Discussion

Patients with AGS have been known to report allergic manifestations associated with the ingestion of dairy products, due to the presence of α-Gal carrying proteins, which have recently been identified in bovine milk whey.[7,19,22,25] In order to prove that these milk-induced allergic reactions are due to IgE recognizing α-Gal, it is necessary to exclude other more common causes of reactions to milk, including lactose intolerance and cow's milk allergy.[35] In the present work, we have found that milk fat globule associated proteins contain α-Gal epitopes recognized by the specific IgE of patients with AGS. Specifically, we have demonstrated, for the first time, that BT, LA, and XO contained in milk fat globules are α-gal glycosylated. The pool of patients' sera also immune-recognized milk LF, LPO, and IgG-like proteins, as expected.

The α-gal-glycosylation of BT, LA, and XO was confirmed by means of immunoblotting experiment, since immunorecognition by the anti-α-gal IgG and by AGS patients' sera was lost after de-glycosylation. The LC-HRMS approach showed that new tryptic peptides containing Asp instead of Asn were generated after enzymatic de-glycosylation giving reason for possible α-gal-glycosylation sites on these proteins. Although the glycosylation sites of BT and LA had previously been identified by Sato et al.[36] and by Hvarregaard et al.[37] we have identified, for the first time, the glycosylation site of XO (Asn_{704} modified to Asp_{704}).

No correlations were found between the levels of α-gal sIgE and the immunoreaction profile when the serum of single patients was tested. This is not surprising, as the presence of elevated IgE levels is indicative of sensitization to α-gal but is not necessarily predictive of a severe allergic reaction.[33]

The role of XO seems to be prevalent, since it was identified in most of the immunoreactive bands, especially those separated in the upper part of the gel where no Comassie Blue stained bands were detectable, but both anti-α-gal IgG antibody and AGS patient sera showed the highest immunoreactivity. For this reason, bovine XO was used to perform immunoinhibition experiments on three selected patients. XO was able to inhibit immunorecognition by the AGS patient sera as well as thyroglobulin, but a higher amount of protein was needed, probably because there are fewer glycosylation sites on XO than on thyroglobulin.

Table 3. Analysis of the xanthine oxidase butyrophilin and lactadherin glycosylation sites by means of LC-HRMS.

	Theoretical data		LC-HRMS experimental data	
α-gal MFGP	*N*-glycosylated triplets	Triplets already known from literature	Peptide-containing triplet before enzymatic de-glycosylation	Peptide-containing modified triplet (N→D)
XO (P80457)	N_{644}ET	Not	Not found	Not found
	N_{704}NS	Not	Not found	**704–713 (D_{704}NS)**
	N_{904}LS	Yes (in goat)	903–912 (N_{904}LS)	Not found
	N_{1073}SS	Yes (in human)	Not found	Not found
	N_{1288}NT	Not	1283–1290 (N_{1288}NT)	Not found
BT (P18892)	N55VS	Yes (in cow)	Not found	Not found
	N215VS	Yes (in cow)	Not found	**215–221 (D_{215}VS)**
	N337MT	Not	Not found	Not found
LA (Q95114)	N_{59}ET	Yes (in cow)	Not found	Not found
	N_{144}NS	Not	138–149 (N_{144}NS)	Not found
	N_{227}NS	Yes (in cow)	Not found	**221–232 (D_{227}NS)**
	N_{390}NS	Not	382–395 (N_{390}NS)	Not found

N: asparagine; D: aspartic acid; MFGP: milk fat globule protein; XO: xanthine oxidase; BT: butyrophilin; LA: lactadherin.

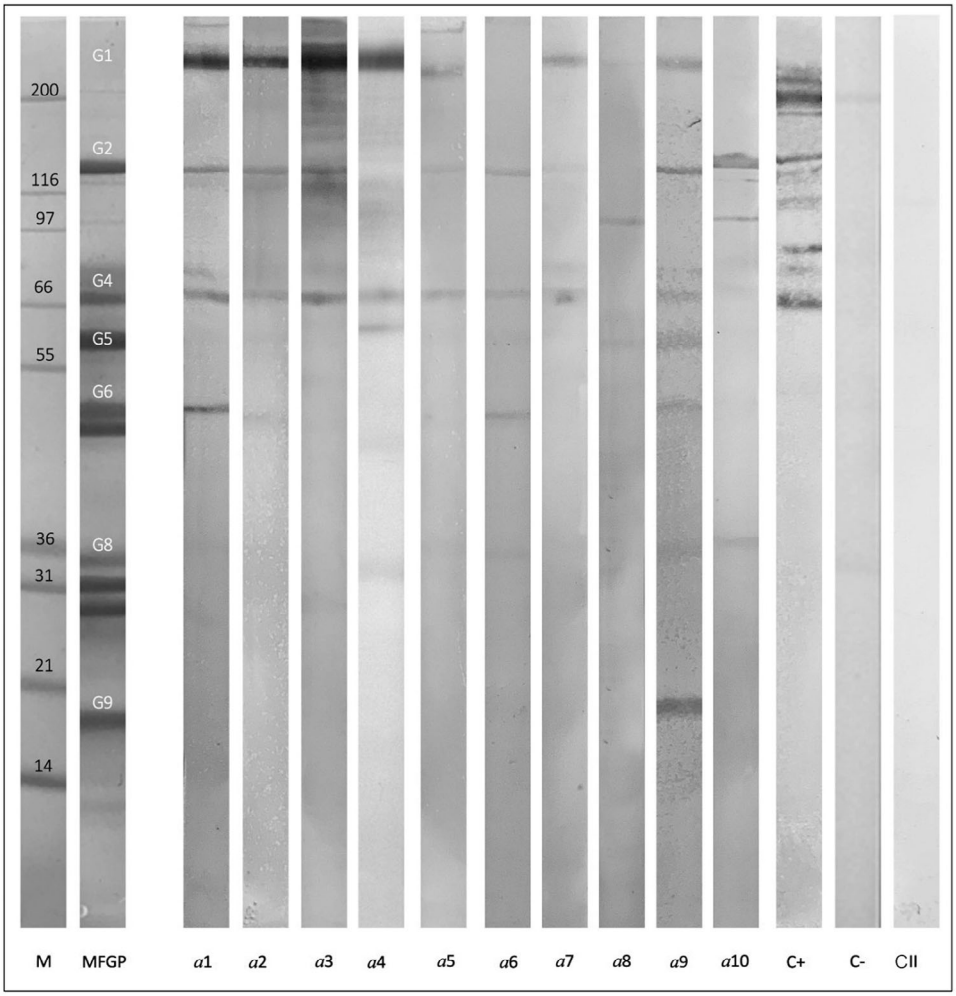

Figure 3. Recognition of milk fat globule associated proteins (MFGP) by α-gal syndrome (AGS) patients. Immunoblotting of MFGP with the sera of 10 AGS patients (from α1 to α10). M: molecular weight marker; C+: thyroglobulin. C−: patient not assuming meat. negative control CII: secondary antibody control.

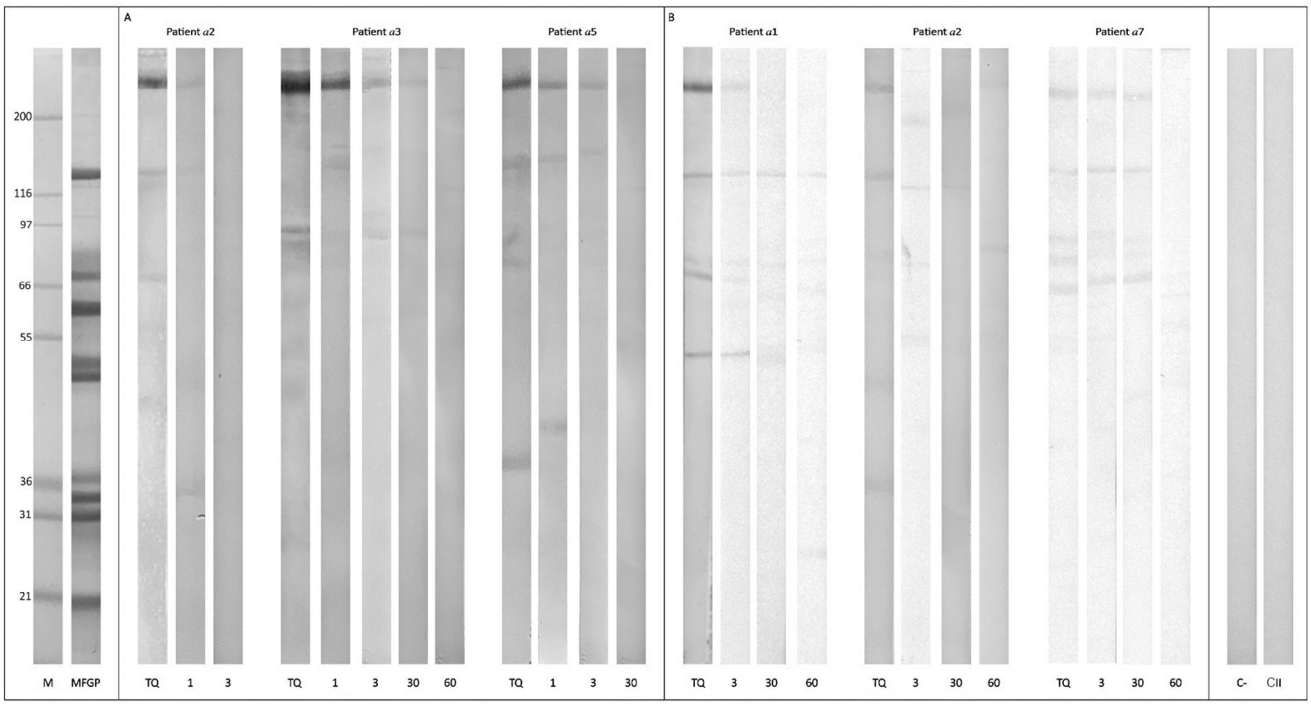

Figure 4. Immunoprecipitation experiments of α-gal syndrome (AGS) patient's sera. A) Immunoblotting of milk fat globule associated proteins (MFGP) with the sera of three patients (α2, α3, and α5) immunoprecipitated with different concentrations of thyroglobulin (1, 3, 30, and 60 µg). B) Immunoblotting of MFGP with the sera of three patients (α1, α2, and α7) immunoprecipitated with different concentrations of bovine xanthine oxidase (3, 30, and 60 µg). M: molecular weight marker; C−: patient not assuming meat; CII: secondary antibody control.

In conclusion, we have found that milk fat globule associated proteins contain α-Gal epitopes recognized by the specific IgE of patients with AGS. Previously, Ròman-Carrasco et al.[16] demonstrated the presence of α-gal determinants in the lipidic fraction of meat and their ability to cross the intestinal monolayer, as well as the potential to trigger allergic reactions in patients with AGS.

The IgEs of all the patients recruited in the present study, recognized several α-Gal carrying proteins contained in whey and in milk fat globules, although those consuming milk and dairy products seem to tolerate them. This is not surprising, as IgE reactivity to bovine milk has been reported in 70–90% of AGS patients (7,21,25), but the allergic manifestations triggered by dairy products only seem to affect at most 20% of patients.[20] Additional host factors are certainly associated with clinical manifestations, and the role of α-Gal carrying glycolipids in reactions to milk and dairy products needs to be further investigated.

4. Experimental Section

Characterization of the Patients: This observational study was carried out on 10 adult Italian AGS patients at the Allergy and Clinical Immunology University Clinic in Turin (AO Ordine Mauriziano di Torino). Details about the characterization of patients enrolled in the study are available in the Online Repository Material and Method and in Table S1 (Supporting Information).

Chemicals: Details pertaining to this topic are available in the Online Repository.

Milk Fat Globule Membrane Associated Protein Extraction: The MFGP was extracted according to Barello et al.[38] The milk protein fractions were extracted from 60 mL of whole fresh cow's milk centrifugated at 5000×g for 30 min at 6 °C to remove somatic cells and impurities. Caseins (CAS), whey proteins (WP), and fat globules were obtained by means of high-speed centrifugation (189 000×*g* for 70 min at 6 °C) and stored at −20 °C.

In order to extract MFGP, 300 µL of extraction buffer (5 mM Tris-HCl pH 8.8; 6.5 M urea; 2.2 M thiourea; 1% w/v ASB-14)[39] was added to the milk fat globule pad (200 µL). The sample was incubated under agitation at room temperature (RT) for 1 h and centrifugated at 21 000×*g* for 30 s. After removing the floating cream layer, the supernatant containing the MFGP was collected and precipitated with methanol and chloroform, as described by Wessel and Flügge,[40] to remove any salts or detergent residues. The protein pellet was quantified by means of 2-D QuantKit.

Glycosylated Milk Fat Globule Membrane Associated Protein (MFGP) Enrichment: Sixty microliters of BioMag Goat Anti-Human IgG beads (5.2 mg mL^{-1}) (BioMag beads) were washed twice with 500 µL of PBS. The washed BioMag beads were blocked twice with TBS with 0.3% of Tween 20 (blocking solution) for 15 min under agitation at 4 °C. After removing the BS, the BioMag beads were incubated with 1:1 of human IgG1 anti α-gal-epitope antibody (α-gal-IgG Ab) or 6 h under rotation at 4 °C. The α-gal-IgG Ab/BioMag bead complexes were collected by means of a magnetic bar and were washed twice with 500 µL of PBS. Sixty micrograms of MFGP were added to the α-gal-IgG Ab/BioMag bead complex and incubated overnight (ON) at 4 °C. The α-gal-IgG antibody/BioMag bead/MFGP complexes were then collected again and washed twice with 500 µL of PBS. The MFGP and α-gal-IgG antibodies were released from the BioMag beads by incubating them with the elution buffer (1% (w/v) SDS, 100 mM Tris HCl, pH 7.4, 10 mM DTT, 8 M urea) for 10 min at 95 °C. The proteins released from the beads were then used in the subsequent experiments.

Milk Fat Globule Membrane Associated Protein N-de-Glycosylation: Enzymatic removal of the N-linked glycans was performed using PNGase F, a glycan-Asn-amidase that specifically cleaves the innermost GlcNAc of all N-linked oligosaccharides, unless they carry α(1–3) core-bound fucose residues.[41] The experiment was carried out under denaturing conditions: 40 g of proteins were resuspended in a modified Laemmli buffer (60 mM

Tris-HCl pH 6.8, 0.25% SDS, 10% glycerol) and 1 uL of 1 M DTT was added. The sample was incubated at 95 °C for 5 min. After cooling, 2 µL of 10% NP-40 and a quantity of PNGase F (10 U μg^{-1}) were added, in a 1:1 enzyme/substrate ratio, to the sample and incubated at 37 °C for 3 h and overnight (ON) under slight shaking.

Protein Separation and LC-HRMS Analysis: The LDS-PAGE separation and LC-HRMS analysis were performed according to Cirrincione et al.[42] Protein separation was performed using Lithium dodecyl sulfate-PAGE (LDS-PAGE) with precast gels (NuPAGE 4–12% Bis–Tris gels) and MES Running buffer in an XCell SureLock Mini–Cell System (Invitrogen), according to the manufacturer's instructions. Each sample was diluted in a NuPage LDS Sample Buffer, under reducing conditions (1% of NuPage sample reducing agent) and loaded in an equal amount (5 µg). LMW standards were run as the molecular weight reference. Gels were stained with Colloidal Coomassie Blue[43] and scanned with a ChemiDoc MP System densitometer (Bio-Rad) at 600 dpi of resolution.

The selected LDS PAGE bands were cut and reduced in 10 mM DTT/50 mM NH_4HCO_3 for 45 min at 56 °C and subsequently alkylated in 55 mM IAA/50 mM NH^4HCO^3 for 30 min at room temperature in the dark. They were then de-stained with ACN 50%/50 mM NH_4HCO_3, pure ACN and, again with ACN 50%/50 mM NH_4HCO_3. The samples were dried in the 5301 Eppendorf Concentrator (Eppendorf, Hamburg, Germany) and digested with 7 µL of modified porcine proteomic grade trypsin (75 ng μL^{-1}) in 25 mM NH_4HCO_3/10% ACN, at 37 °C, ON, under shaking. One microliter of 5% FA was added to stop the enzymatic protein digestion. The Orbitrap Q Exactive Plus, coupled to a UHPLC binary pump system (Vanquish Thermo Fisher Scientific, Waltham, Massachusetts, USA), was used to perform protein identification. The stationary phase was a BioBasic C18 HPLC Column (1 × 150 mm, 5 µm; Thermo Scientific). The mobile phases were 0.1% (v/v) FA in MilliQ water (A) and 0.1% (v/v) FA in ACN (B), and they were eluted at a flowrate of 50.0 µL min^{-1} with increasing concentrations of solvent B, from 5% to 70%, over 50 min and with 80% for 5 min. The oven temperature was set at 55 °C. The autosampler was set at 6 °C. The injection volume was 4.0 µL. Mass spectra were acquired in Full MS-ddMS2 mode. The instrument was set up so that Full MS spectra were acquired in an m/z scan range of 150–1800, the resolution was set at 70 000, the maximum IT was 200 ms, the AGC target was 5×10^5, and the charge exclusion was unassigned. Up to 12 of the most intense ions in MS1 were selected for fragmentation in the MS/MS mode. The fragmentation spectra resolution was set at 17 500 for the MS/MS spectra, with a dynamic exclusion of 20 s and an isolation window of 2.0 m/z, while the normalized collision energy was set at 28, the maximum IT at 200 ms and the AGC target at 2×10^4.

Protein Identification Strategy: All the Data Dependent Analysis (DDA) files were searched using MaxQuant (https://maxquant.org) v. 2.0.3.0 against the UniProt *Bos taurus* database (reviewed and unreviewed). The search was performed using a list of contaminants devoid of bovine proteins, because they were the target. The search parameters were set as follow: S-carbamidomethyl derivate on cysteine as a fixed modification, oxidation on methionine, Acetyl (N-term) as variable modifications and two missed cleavage sites for trypsin digestion. The possibility of Asn becoming Asp was added as a variable modification for bands derived from enzymatic de-glycosylation. The MS/MS fragment mass tolerance was set at 20 ppm. A minimum of 3 peptides, an FDR of 0.01% for both the protein and peptides, and a score of 20 for unmodified and modified peptides were set for the protein identification. Only proteins identified with a score >35 were listed in the tables, with the exception of the identification performed on unstained bands cut in the upper part of the gels where a score of >10 was allowed.

Whey and Milk Fat Globule Membrane Associated Protein Immunoblotting: After LDS-PAGE, the protein bands were electro-transferred into Nitrocellulose Membranes (0.2 µm) with an XCell II Blot Module, using a transfer buffer with 10% methanol (v/v). The membranes were blocked in TBS with 0.3% Tween 20 (blocking solution) for 30 min and incubated ON at 4 °C with 800 µL of the HRP conjugated Human IgG1 anti α-Gal epitope antibody (Absolute Antibody) diluted 1:1000 in the incubation buffer (TBS, 0.05% Tween 20, 0.05% vegetal gelatin) or with the patients' sera diluted 1:10 in the incubation buffer. After incubation, the membranes were washed three times with TBS, 0.05% and Tween 20 (washing solution) for 10 min. The membranes incubated with the patient's sera were incubated again with the anti-Human IgE antibody (Sera Care Life Sciences Inc.) diluted 1:5000 in the incubation buffer. The membranes were washed three times and developed with different development kits according to the used primary antibody: an Alkaline Phosphatase Substrate Kit (Bio-Rad) for the patients' sera and an Opti 4 CN Kit (Bio-Rad) for the HRP conjugated Human IgG1 anti α-Gal epitope antibody.

Immunoprecipitation of the AGS Patient Sera: Immunoprecipitation experiments were performed with two glycosylated proteins: bovine thyroglobulin and the bovine xanthine oxidase (XO) from Sigma-Aldrich. The sera of three AGS patients (α2, α3, and α5) were incubated for 1 h at room temperature with four amounts of thyroglobulin (1, 3, 30, and 60 µg) and other three patients (α1, α2, and α7) were incubated at the same conditions with three amounts of xanthine oxidase (3, 30, and 60 µg). Nitrocellulose membranes containing electro-transferred MFGP were blocked with the blocking solution for 30 min and then incubated overnight at 4 °C with the immunoprecipitated sera. The immunoblotting procedure was then performed as previously explained in see Section Protein Identification Strategy.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

Data available on request from the authors.

Keywords

alpha-gal carbohydrate, alpha-gal syndrome, food allergy, milk, xanthine oxidase

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[1] S. P. Commins, S. M. Satinover, J. Hosen, J. Mozena, L. Borish, B. D. Lewis, J. A. Woodfolk, T. A. Platts-Mills, *J. Allergy Clin. Immunol.* **2009**, *123*, 426.e2.
[2] J. Fischer, A. S. Yazdi, T. Biedermann, *Allergo J. Int.* **2016**, *25*, 55.
[3] D. Apostolovic, J. Mihailovic, S. P. Commins, M. Wijnveld, M. Kazimirova, M. Starkhammar, H. Stockinger, T. A. E. Platts-Mills, T. Cirkovic Velickovic, C. Hamsten, M. van Hage, *Allergy* **2020**, *75*, 217.
[4] L. Boussamet, E. Montassier, J. P. Soulillou, L. Berthelot. *Clinical Immunology*, Vol. 235, Academic Press Inc., Cambridge, USA, **2022**.
[5] J. L. Kennedy, A. P. Stallings, T. A. E. Platts-Mills, W. M. Oliveira, L. Workman, H. R. James, A. Tripathi, C. J. Lane, L. Matos, P. W. Heymann, S. P. Commins, *Pediatrics* **2013**, *131*, e1545.
[6] I. Young, C. Prematunge, K. Pussegoda, T. Corrin, L. Waddell, *Ticks Tick Borne Dis.* **2021**, *12*, 101674.
[7] M. B. G. Kiewiet, D. Apostolovic, M. Starkhammar, J. Grundström, C. Hamsten, M. van Hage, *J. Allergy Clin. Immunol.: Pract.* **2020**, *8*, 2027.e2.

[8] S. van Nunen, *Curr. Treat. Options Allergy* **2014**, *1*, 262.
[9] J. M. Thompson, A. Carpenter, J. Gilbert Kersh, T. Wachs, S. P. Commins, J. S. Salzer, *Morbidity and Mortality Weekly Report Geographic Distribution of Suspected Alpha-gal Syndrome Cases-United States* [Internet], Centers for Disease Control and Prevention | MMWR, Vol. *72*, CDC - Atlanta, USA, **2023**. Available from: https://ndc.services.cdc.gov/case-definitions/alpha-gal-syndrome-ags/
[10] D. Apostolovic, M. Krstic, J. Mihailovic, M. Starkhammar, T. Cirkovic Velickovic, C. Hamsten, M. van Hage, *Sci. Rep.* **2017**, *7*, 5201.
[11] A. Gonzalez-Quintela, A. S. Dam Laursen, C. Vidal, T. Skaaby, F. Gude, A. Linneberg, *Clin. Exp. Allergy* **2014**, *44*, 1061.
[12] M. B. Mateo-Borrega, B. Garcia, C. H. Larramendi, J. Azofra, E. González-Mancebo, M. I. Alvarado, M. D. Alonso Díaz de Durana, R. Núñez Orjales, M. C. Diéguez, M. Guilarte, A. M. Soriano Galarraga, G. Sosa, A. Ferrer, A. García Moral, A. M. Beristain, J. Bartra, *J. Invest. Allergol. Clin. Immunol.* **2019**, *29*, 436.
[13] D. Villalta, L. Cecchi, A. Farsi, F. Chiarini, P. Minale, S. Voltolini, E. Scala, O. Quercia, L. Muratore, V. Pravettoni, A. M. Calamari, G. Cortellini, R. Asero, *Eur. Ann. Allergy Clin. Immunol.* **2017**, *49*, 263.
[14] T. A. E. Platts-Mills, S. P. Commins, T. Biedermann, M. van Hage, M. Levin, L. A. Beck, M. Diuk-Wasser, U. Jappe, D. Apostolovic, M. Minnicozzi, M. Plaut, J. M. Wilson, *J. Allergy Clin. Immunol.* **2020**, *145*, 1061.
[15] O. I. Iweala, S. K. Choudhary, C. T. Addison, C. J. Batty, C. M. Kapita, C. Amelio, A. J. Schuyler, S. Deng, E. M. Bachelder, K. M. Ainslie, P. B. Savage, P. J. Brennan, S. P. Commins, *J. Allergy Clin. Immunol.* **2020**, *146*, 450.
[16] P. Román-Carrasco, B. Lieder, V. Somoza, M. Ponce, Z. Szépfalusi, D. Martin, W. Hemmer, I. Swoboda, *Allergy* **2019**, *74*, 1956.
[17] N. Chakrapani, J. Fischer, K. Swiontek, F. Codreanu-Morel, F. Hannachi, M. Morisset, C. Mugemana, D. Bulaev, S. Blank, C. Bindslev-Jensen, T. Biedermann, M. Ollert, C. Hilger, *J. Allergy Clin. Immunol.* **2022**, *150*, 396.e11.
[18] A. S. Carson, A. Gardner, O. I. Iweala, *J. Immunol.* **2022**, *208*, 267.
[19] S. P. Commins, *Curr. Allergy Asthma Rep.* **2016**, *16*, 61.
[20] P. Armstrong, A. Binder, C. Amelio, G. Kersh, B. Biggerstaff, C. Beard, L. Petersen, S. Commins, *J. Allergy Clin. Immunol.* **2020**, *145*, AB145.
[21] J. M. Wilson, A. J. Schuyler, L. Workman, M. Gupta, H. R. James, J. Posthumus, E. C. McGowan, S. P. Commins, T. A. E. Platts-Mills, *J. Allergy Clin. Immunol.: Pract.* **2019**, *7*, 2348.e4.
[22] S. P. Commins, *Expert Rev. Clin. Immunol.* **2020**, *16*, 667.
[23] G.-S. Choi, J.-H. Kim, Y.-S. Shin, D.-H. Nahm, H.-S Park, *Allergy* **2010**, *65*, 1065.
[24] R. J. Mullins, H. James, T. A. E. Platts-Mills, S. Commins, *J. Allergy Clin. Immunol.* **2012**, *129*, 1334.
[25] M. Perusko, D. Apostolovic, M. B. G. Kiewiet, J. Grundström, C. Hamsten, M. Starkhammar, T. Cirkovic Velickovic, M. van Hage, *Allergy* **2021**, *76*, 3766.
[26] A. German-Sanchez, A. Alonso-Llamazares, M. Latorre-Ibañez, B. Bartolome-Zavala, *J. Invest. Allergol. Clin. Immunol.* **2023**, *33*, 491.
[27] L. R. Brink, B. Lönnerdal, *J. Nutr. Biochem.* **2018**, *58*, 131.
[28] B. Guan, Z. Zhang, Y. Chai, X. Amantai, X. Chen, X. Cao, X. Yue, *Trends in Food Science and Technology*, ELSEVIER, Amsterdam, The Netherlands **2022**, Vol. *128*, p. 1.
[29] M. Manoni, D. Cattaneo, S. Mazzoleni, C. Giromini, A. Baldi, L. Pinotti, *Dairy*, MDPI, Basel, Switzerland Vol. *2*, **2021**. p. 202.
[30] S. Spertino, V. Cipriani, C. De Angelis, M. G. Giuffrida, F. Marsano, M. Cavaletto, *Mol. BioSyst.* **2012**, *8*, 967.
[31] T. A. Reinhardt, J. D. Lippolis, *J. Dairy Res.* **2006**, *73*, 406.
[32] Y. Yang, N. Zheng, W. Wang, X. Zhao, Y. Zhang, R. Han, L. Ma, S. Zhao, S. Li, T. Guo, C. Zang, J. Wang, *Proteomics* **2016**, *16*, 2792.
[33] J. M. Wilson, L. Erickson, M. Levin, S. M. Ailsworth, S. P. Commins, T. A. E. Platts-Mills, *Allergy: European Journal of Allergy and Clinical Immunology*, WILEY, Hoboken, USA **2024**.
[34] C. E. Loprinzi Brauer, M. S. Motosue, J. T. Li, J. B. Hagan, M. F. Bellolio, S. Lee, R. L. Campbell, *J. Allergy Clin. Immunol.: Pract.* **2016**, *4*, 1220.
[35] A. M. Binder, D. Cherry-Brown, B. J. Biggerstaff, E. S. Jones, C. L. Amelio, C. B. Beard, L. R. Petersen, G. J. Kersh, S. P. Commins, P. A. Armstrong, *Allergy* **2023**, *78*, 477.
[36] T. Sato, K. Takio, A. Kobata, D. E. Greenwalt, K. Furukawa, *J. Biochem.* **1995**, *117*, 147.
[37] J. Hvarregaard, M. H. Andersen, L. Berglund, J. T. Rasmussen, T. E. Petersen, *Eur. J. Biochem.* **1996**, *240*, 628.
[38] C. Barello, L. P. Garoffo, G. Montorfano, S. Zava, B. Berra, A. Conti, M. G. Giuffrida, *Mol. Nutr. Food Res.* **2008**, *52*, 1448.
[39] M. P. Molloy, N. D. Phadke, H. Chen, R. Tyldesley, D. E. Garfin, J. R. Maddock, P. C. Andrews, *Proteomics* **2002**, *2*, 899.
[40] D. Wessel, U. I. Flügge, Anal. Biochem. **1984** [cited 2020 Mar 17], *138*, 141. Available from: https://www.sciencedirect.com/science/article/abs/pii/0003269784907826
[41] V. Tretter, F. Altmann, L. März, *Eur. J. Biochem.* **1991**, *199*, 647.
[42] S. Cirrincione, B. Aiuto, E. Gosso, C. Schiavone, C. Portesi, A. M. Rossi, G. Monti, L. Cavallarin, C. Lamberti, G. M. Giuffrida, *J. Food Compos. Anal.* **2023**, *121*, 105386.
[43] G. Candiano, M. Bruschi, L. Musante, L. Santucci, G. M. Ghiggeri, B. Carnemolla, P. Orecchia, L. Zardi, P. G. Righetti, *Electrophoresis* **2004**, *25*, 1327.