POLITECNICO DI TORINO
Repository ISTITUZIONALE

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

Original
Milk Fat Globule Proteins Are Relevant Bovine Milk Allergens in Patients with alpha-Gal Syndrome / Aiuto, B., Cirrincione, S., Giuffrida, M.G., Cavallarin, L., Portesi, C., Rossi, A.M., Borreani, G., Rolla, G., Geuna, M., Nicola, S., Quinternetto, A., Alessi, L., Saracco, E., Brussino, L., Lamberti, Cristina.. - In: MOLECULAR NUTRITION & FOOD RESEARCH. - ISSN 1613-4125. - STAMPA. - 68:10(2024). [10.1002/mnfr.202300796]

Availability:
This version is available at: 11583/2989925 since: 2024-09-02T09:51:32Z

Publisher:
Wiley

Published
DOI:10.1002/mnfr.202300796

Terms of use:

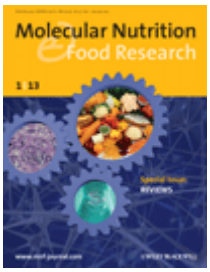
This article is made available under terms and conditions as specified in the corresponding bibliographic description in the repository

Publisher copyright
AAAS preprint/submitted version e/o post-print Author Accepted Manuscript

preprint/submitted version e/o post-print Author Accepted Manuscript

(Article begins on next page)

26 November 2026



Milk fat globule proteins are relevant bovine milk allergens in patients with α-Gal syndrome

Journal:	*Molecular Nutrition and Food Research*
Manuscript ID	Draft
Wiley - Manuscript type:	Research Article
Date Submitted by the Author:	n/a
Complete List of Authors:	Aiuto, Beatrice; Institute of Sciences of Food Production National Research Council Torino Unit Cirrincione, Simona; Institute of Sciences of Food Production National Research Council Torino Unit, Giuffrida, Maria Gabriella; Institution of Science of Food Production National Research Council, Torino Unit, Grugliasco (TO) Cavallarin, Laura; Institution of Science of Food Production National Research Council, Torino Unit, Grugliasco (TO) Portesi, Chiara; National Metrology Institute of Italy Rossi, Andrea Mario; National Metrology Institute of Italy Borreani, Giorgio; University of Turin Rolla, Giovanni; Azienda Ospedaliera Ordine Mauriziano di Torino Geuna, Massimo; Azienda Ospedaliera Ordine Mauriziano di Torino Nicola, Stefania; Azienda Ospedaliera Ordine Mauriziano di Torino Quinternetto, Anna; Azienda Ospedaliera Ordine Mauriziano di Torino Alessi, Lucrezia; Azienda Ospedaliera Ordine Mauriziano di Torino Saracco, Elena; Azienda Ospedaliera Ordine Mauriziano di Torino Brussino, Luisa; Azienda Ospedaliera Ordine Mauriziano di Torino Lamberti, Cristina; Institution of Science of Food Production National Research Council, Torino Unit, Grugliasco (TO)
Keywords:	alpha-gal syndrome, alpha-gal carbohydrate, food allergy, milk, xanthine oxidase

SCHOLARONE™
Manuscripts

Title

Milk fat globule proteins are relevant bovine milk allergens in patients with α-Gal syndrome.

Beatrice Aiuto[a,b], Simona Cirrincione[a]*, Maria Gabriella Giuffrida[a], Laura Cavallarin[a], Chiara Portesi[c], Andrea Mario Rossi[c], Giorgio Borreani[d], Giovanni Rolla[e], Massimo Geuna[e], Stefania Nicola[e], Anna Quinternetto[e], Lucrezia Alessi[e], Elena Saracco[e], Luisa Brussino[e], Cristina Lamberti[a].

Institutional affiliation

a. Institute of the Science of Food Production (ISPA) – National Research Council, Largo Braccini 2, 10095 Grugliasco, TO, Italy
b. Politecnico di Torino, Corso Castelfilardo 39, 10129 Torino, Italy
c. National Institute of Metrological Research (INRIM), Strada delle Cacce 91, 10135 Torino, Italy
d. University of Turin, Italy - Department of Agriculture, Forestry and Food Sciences (DISAFA)
e. Department of Medical Sciences, Allergy and Clinical Immunology Unit, University of Torino & Mauriziano Hospital, Torino, Italy

***Corresponding author**

Simona Cirrincione:

simona.cirrincione@ispa.cnr.it

Largo Braccini, 2

10095 Grugliasco (TO), Italy

For Peer Review

+39 0116709229

Abbreviations: alpha-gal syndrome (AGS); galactose-α-1,3-galactose (α-gal); bovine γ-globulin (BGG); lactoferrin (LF); lactoperoxidase (LPO); milk fat globule protein (MFGP); Xanthine Oxidase (XO); butyrophilin (BT); lactadherin (LA); whey proteins (WP); caseins (CAS).

Kye words: alpha-gal syndrome, alpha-gal carbohydrate, food allergy, milk, xanthine oxidase.

For Peer Review

Abstract

Scope. 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 whey milk proteins. Considering the already described role of the lipid fraction of meat in AGS clinical manifestations, the aim of this work has been to investigate whether the milk fat globule proteins (MFGP) could be involved in AGS.

Methods and results. The MFGP were extracted and their recognition by the IgE of AGS patients was proved through immunoblotting experiments with the sera of AGS patients. The identification of the immunoreactive proteins by LC-HRMS analysis allowed to demonstrate for the first time that butyrophillin, lactoadherin and xanthine oxidase are α-gal glycosylated. The role of xanthine oxidase seems to be prevalent since both the anti-α-gal antibody and AGS patient sera showed the highest immunoreactivity against it.

Conclusion. The results obtained in this study have confirmed the role of α-Gal carrying glycoproteins in AGS patients reacting to milk. Although additional factors are probably associated with the clinical manifestations, the consumption of milk and milk products should be limited or even avoided in individuals with AGS.

For Peer Review

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, most 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 are also produced and are responsible for 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 to 6 hours 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 5000 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 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, 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

For Peer Review

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 might contain α-Gal-epitopes, although in smaller amounts.[19] Some recent studies,[7,20,21] including one involving the analysis of a large cohort of 2,500 AGS patients in the USA,[22] have proved 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. Perusko et al.[24] demonstrated that bovine γ-globulin (BGG), lactoferrin (LF), and lactoperoxidase (LPO) are α-Gal carrying proteins that have been recognized by the IgE 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.[25]

Considering the involvement of milk proteins in AGS and the role of the lipid fraction in facilitating clinical manifestations of AGS, the aim of this work has been to investigate whether the milk fat globule protein (MFGP) fraction could also play a role in AGS.

2. Experimental Section

2.1 Characterization of the patients

For Peer Review

This observational study was carried out on 10 adults Italian AGS patients at the Allergy and Clinical Immunology University Clinic in Turin (AO Ordine Mauriziano di Torino). All the patients were diagnosed with AGS based on the history of at least one previous hypersensitivity reaction to mammalian meat and/or its related products (food gelatine), and the presence of positive α-Gal specific IgE antibodies. Only adult patients (≥18 years old) with an established diagnosis of AGS were enrolled. The exclusion criteria were age<18 years old, ongoing anti-IgE biological therapy (Omalizumab), or the lack of informed consent release. The study was approved by the local ethical committee (Comitato Etico Interaziendale A.O.U. Città della Salute e della Scienza di Torino- A.O. Ordine Mauriziano- A.S.L. Città di Torino, *study number 0053278, date of approval: 24.05.2019*) and conducted according to the Declaration of Helsinki. Data were collected between June 2019 and March 2023.

The demographic data, the description of previous reactions (culprit food, clinical presentation, time of symptoms onset, treatment, and the presence of co-factors) and their history of previous tick bites are reported in Table 1. All the patients underwent blood tests to analyze the total serum IgE and α-Gal specific IgE antibodies (Immunocap Fluorescence Enzyme Immunoassay Feia, by Thermo Fisher). According to the manufacturer's recommendations, levels of total IgE below 205 KU/L and specific IgE below 0.10 KUA/L, respectively, were considered normal. Serum tryptase (Immunocap Fluorescence Enzyme Immunoassay Feia, supplied by Thermo Fisher) was also assessed in all the patients, and levels below 11,4 µg/l were considered normal.

A statistical analysis was performed, using IBM SPSS Statistics for Windows, version 26 (IBM Corp., Armonk, NY, USA). A normality distribution of data was first tested using the Kolmogorov–Smirnov normality test, and a descriptive analysis of the variables was then performed. The baseline characteristics were evaluated over the whole cohort and expressed as the mean (standard

For Peer Review

deviation, SD), unless otherwise specified, for the continuous variables, and as absolute and relative frequencies for the categorical variables.

2.2 Chemicals

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

2.3 Milk fat globule membrane associated protein extraction

The MFGP was extracted according to Barello et al.[26] Details are available in the Online Repository.

2.4 Glycosylated milk fat globule membrane associated protein enrichment

Sixty µl of BioMag Goat Anti-Human IgG beads (5,2mg/ml) (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 minutes under agitation at 4°C. After removing the BS, the BioMag beads were incubated with 1:1 of α-gal-IgG Ab for 6 hours 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 µg of MFGP were added to the α-gal-IgG Ab /BioMag bead complexe and incubated overnight (O.N.) 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, 8M urea) for 10 min at 95°C. The proteins released from the beads were then used in the subsequent experiments.

2.5 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–

For Peer Review

3) core-bound fucose residues.[27] The experiment was carried out under denaturing conditions: 40g of proteins were resuspended in a modified Laemmli buffer (60mM Tris-HCl pH 6.8, 0,25% SDS, 10% glycerol) and 1ul of 1M 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 (10U/µg) were added, in a 1:1 enzyme/substrate ratio, to the sample and incubated at 37 °C for 3 hours and overnight (ON) under slight shaking.

2.6 Protein separation and LC-HRMS analysis

The LDS-PAGE separation and LC-HRMS analysis were performed according to Cirrincione et al.[28] Details are available in the Online Repository.

2.7 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 our 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 2 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 >30 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.

2.8 Whey and milk fat globule membrane associated protein immunoblotting

For Peer Review

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., Milford, Massachusetts) 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.

2.9 Immunoprecipitation of the AGS patient sera

Immunoprecipitation experiments were performed with two glycosylated proteins: bovine thyroglobulin and the bovine xanthine oxidase from Sigma-Aldrich. The sera of three AGS patient (α2, α3, α5) were incubated for 1h at room temperature with four amounts of thyroglobulin (1, 3, 30, 60 µg) and other three patients (α1, α2, α7) were incubated at the same conditions with three amounts of xanthine oxidase (3, 30, 60 µg). Nitrocellulose membranes containing electro-transferred MFGP associated proteins 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 Section 2.7.

3. Results

For Peer Review

3.1 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) with a history of non-IgE mediated milk hypersensitivity reaction was used as a healthy control.

3.1.1 Comorbidities

Three patients had arterial hypertension, one suffered from diabetes, one was HIV positive, and one suffered from atrial fibrillation. All the patients had a normal weight, and three of them were smokers. As far as atopic diseases are concerned, 3 patients had allergic rhinitis, 2 patients showed sensitization to lipid transfer protein (LTP) with mild food allergy manifestations, and one patient reported nonsteroidal anti-inflammatory drug (NSAID) hypersensitivity (urticaria) as well ampicillin hypersensitivity. One patient (male, 64 years old, α6) was affected by systemic indolent mastocytosis.

3.1.2 Clinical presentation of AGS

All the AGS patients reported at least one delayed reaction (average 3.40± 1.58 events/person) with a mean onset time of 4.1 hours after eating red meat, innards, or meat-related food (Tab. 1). None of the patients experienced reactions to cow's milk or dairy products. The most common culprit food was pork meat. Urticaria was the most common clinical manifestation (100%), followed by gastrointestinal symptoms (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.[29] 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

For Peer Review

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 ± 35.87 KUA/l) with a mean serum total IgE of 389.99±429.94 KU/l. Tryptase resulted normal in all the patients, with a mean value of 7,18±3.78 µg/l).

3.1.3 AGS treatment

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.

3.2 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 (Fig 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 (Fig 1, panel B). LC-HRMS analysis (Tab. 2) allowed LF and LPO to be identified in band W3, and several Ig-like domain containing proteins were identified in bands W2, W3, W5, 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

For Peer Review

(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 (Fig. 1, panel C).

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.

3.3 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 (Fig. 2, panel A, 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 de-glycosylation. 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 (Fig. 2, Panel A, lane MFGPbDEG). This reactivity completely disappeared only after a more exhaustive overnight PNGase F de-glycosylation (Fig. 2, panel A, lane MFGPbDEGon). The analysis of the bands containing the N-de-glycosylated proteins that lost reactivity revealed which asparagine carried the α-gal moiety (Fig. 2 panel A and 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 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}

For Peer Review

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.

3.4 The AGS patients' IgE antibodies recognize Xanthine Oxidase and Butirophilin

The MFGP sample was also incubated with the serum of each single patient (Fig. 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, α5) was performed with four concentrations of bovine thyroglobulin (1, 3, 30, 60 μg) (Fig. 4, Panel A). 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, α7) (Fig. 4, Panel B). 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.

4. 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.[7,19,22,24] 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

For Peer Review

to milk, including lactose intolerance and cow's milk allergy.[30] 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 an LC-HRMS approach, since new tryptic peptides containing Asp instead of Asn were generated after enzymatic de-glycosylation, and by means of immunoblotting experiment, since immunorecognition by the anti-α-gal IgG and by AGS patients' sera was lost after de-glycosylation. Although the glycosylation sites of BT and LA had previously been identified by Sato et al.[31] and by Hvarregaard et al.,[32] 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 immuno reaction 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. In fact, an allergic manifestation recognizes several triggers that can exacerbate or mask the reaction itself, thus giving rise to profoundly different clinical pictures.

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, and a smaller number of α-gal-glycosylated sites was indicated for XO than for thyroglobulin.

For Peer Review

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 milk 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 [7,21,24], but the allergic manifestations triggered by dairy products only seem to affect about one third 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.

Conflict of interest statement: the authors have no conflicts of interest to disclose.

5. References

[1] Commins SP, Satinover SM, Hosen J, Mozena J, Borish L, Lewis BD, et al. Delayed anaphylaxis, angioedema, or urticaria after consumption of red meat in patients with IgE antibodies specific for galactose-α-1,3-galactose. Journal of Allergy and Clinical Immunology. 2009;123(2):426-433.e2.

[2] Fischer J, Yazdi AS, Biedermann T. Clinical spectrum of α-Gal syndrome: From immediate-type to delayed immediate-type reactions to mammalian innards and meat. Allergo J Int. 2016;25(2):55–62.

[3] Apostolovic D, Mihailovic J, Commins SP, Wijnveld M, Kazimirova M, Starkhammar M, et al. Allergenomics of the tick Ixodes ricinus reveals important α-Gal–carrying IgE-binding proteins in red meat allergy. Allergy: European Journal of Allergy and Clinical Immunology. 2020;75(1):217–20.

For Peer Review

[4] Boussamet L, Montassier E, Soulillou JP, Berthelot L. Anti α1-3Gal antibodies and Gal content in gut microbiota in immune disorders and multiple sclerosis. Vol. 235, Clinical Immunology. Academic Press Inc.; 2022.

[5] Kennedy JL, Stallings AP, Platts-Mills TAE, Oliveira WM, Workman L, James HR, et al. Galactose-α-1, 3-galactose and delayed anaphylaxis, angioedema, and urticaria in children. Pediatrics. 2013;131(5).

[6] Young I, Prematunge C, Pussegoda K, Corrin T, Waddell L. Tick exposures and alpha-gal syndrome: A systematic review of the evidence. Ticks Tick Borne Dis. 2021;12(3):101674.

[7] Kiewiet MBG, Apostolovic D, Starkhammar M, Grundström J, Hamsten C, van Hage M. Clinical and Serological Characterization of the α-Gal Syndrome—Importance of Atopy for Symptom Severity in a European Cohort. Journal of Allergy and Clinical Immunology: In Practice. 2020;8(6):2027-2034.e2.

[8] van Nunen S. Galactose-Alpha-1,3-Galactose, Mammalian Meat and Anaphylaxis: A World-Wide Phenomenon? Curr Treat Options Allergy. 2014;1(3):262–77.

[9] Cabezas-Cruz A, Hodžić A, Román-Carrasco P, Mateos-Hernández L, Duscher GG, Sinha DK, et al. Environmental and molecular drivers of the α-Gal syndrome. Front Immunol. 2019;10(MAY):1–12.

[10] Apostolovic D, Krstic M, Mihailovic J, Starkhammar M, Cirkovic Velickovic T, Hamsten C, et al. Peptidomics of an in vitro digested α-Gal carrying protein revealed IgE-reactive peptides. Sci Rep. 2017;7(1):1–10.

[11] Gonzalez-Quintela A, Dam Laursen AS, Vidal C, Skaaby T, Gude F, Linneberg A. IgE antibodies to alpha-gal in the general adult population: Relationship with tick bites, atopy, and cat ownership. Clinical and Experimental Allergy. 2014;44(8):1061–8.

[12] Mateo-Borrega MB, Garcia B, Larramendi CH, Azofra J, González-Mancebo E, Alvarado MI, et al. Ige-mediated sensitization to galactose-α-1,3-galactose (α-gal) in urticaria and anaphylaxis in spain: Geographical variations and risk factors. J Investig Allergol Clin Immunol. 2019;29(6):436–43.

For Peer Review

[13] Villalta D, Cecchi L, Farsi A, Chiarini F, Minale P, Voltolini S, et al. Galactose-α-1,3-galactose syndrome: An Italian survey. Eur Ann Allergy Clin Immunol. 2017;49(6):263–9.

[14] Platts-Mills TAE, Commins SP, Biedermann T, van Hage M, Levin M, Beck LA, et al. On the cause and consequences of IgE to galactose-α-1,3-galactose: A report from the National Institute of Allergy and Infectious Diseases Workshop on Understanding IgE-Mediated Mammalian Meat Allergy. Journal of Allergy and Clinical Immunology. 2020;145(4):1061–71.

[15] Iweala OI, Choudhary SK, Addison CT, Batty CJ, Kapita CM, Amelio C, et al. Glycolipid-mediated basophil activation in alpha-gal allergy. Journal of Allergy and Clinical Immunology. 2020;146(2):450–2.

[16] Román-Carrasco P, Lieder B, Somoza V, Ponce M, Szépfalusi Z, Martin D, et al. Only α-Gal bound to lipids, but not to proteins, is transported across enterocytes as an IgE-reactive molecule that can induce effector cell activation. Allergy: European Journal of Allergy and Clinical Immunology. 2019;74(10):1956–68.

[17] Chakrapani N, Fischer J, Swiontek K, Codreanu-Morel F, Hannachi F, Morisset M, et al. α-Gal present on both glycolipids and glycoproteins contributes to immune response in meat-allergic patients. Journal of Allergy and Clinical Immunology. 2022;150(2):396-405.e11.

[18] Carson AS, Gardner A, Iweala OI. Where's the Beef? Understanding Allergic Responses to Red Meat in Alpha-Gal Syndrome. The Journal of Immunology. 2022;208(2):267–77.

[19] Commins SP. Invited Commentary: Alpha-Gal Allergy: Tip of the Iceberg to a Pivotal Immune Response. Curr Allergy Asthma Rep. 2016;16(9):1–3.

[20] Armstrong P, Binder A, Amelio C, Kersh G, Biggerstaff B, Beard C, et al. Descriptive Epidemiology of Patients Diagnosed with Alpha-gal Allergy — 2010–2019. Journal of Allergy and Clinical Immunology. 2020;145(2):AB145.

For Peer Review

[21] Wilson JM, Schuyler AJ, Workman L, Gupta M, James HR, Posthumus J, et al. Investigation into the α-Gal Syndrome: Characteristics of 261 Children and Adults Reporting Red Meat Allergy. Journal of Allergy and Clinical Immunology: In Practice. 2019;7(7):2348-2358.e4.

[22] Commins SP. Diagnosis & management of alpha-gal syndrome:lessons from 2,500 patients. Expert Rev Clin Immunol. 2020;16(7):667–77.

[23] Choi JO, Lee MH, Park HY, Jung SC. Characterization of Fabry mice treated with recombinant adeno-associated virus 2/8-mediated gene transfer. J Biomed Sci. 2010;17(1):1–10.

[24] Perusko M, Apostolovic D, Kiewiet MBG, Grundström J, Hamsten C, Starkhammar M, et al. Bovine γ-globulin, lactoferrin, and lactoperoxidase are relevant bovine milk allergens in patients with α-Gal syndrome. Allergy: European Journal of Allergy and Clinical Immunology. 2021;76(12):3766–75.

[25] German-Sanchez A, Alonso-Llamazares A, Latorre-Ibañez M, Bartolome-Zavala B, Antepara-Ercoreca I. Sheep cheese allergy in Alpha-gal Syndrome. J Investig Allergol Clin Immunol. 2023;33(6):1–7.

[26] Barello C, Garoffo LP, Montorfano G, Zava S, Berra B, Conti A, et al. Analysis of major proteins and fat fractions associated with mare's milk fat globules. Mol Nutr Food Res. 2008;52(12):1448–56.

[27] TRETTER V, ALTMANN F, MÄRZ L. Peptide-N4-(N-acetyl-β-glucosaminyl) asparagine amidase F cannot release glycans with fucose attached α1 → 3 to the asparagine-linked N-acetylglucosamine residue. Eur J Biochem. 1991;199(3):647–52.

[28] Cirrincione S, Aiuto B, Gosso E, Schiavone C, Portesi C, Rossi AM, et al. Proteomic study of walnut oleosome and first evidence on oleosin sensitization in allergic patients. Journal of Food Composition and Analysis. 2023;121(March):105386.

[29] Loprinzi Brauer CE, Motosue MS, Li JT, Hagan JB, Bellolio MF, Lee S, et al. Prospective Validation of the NIAID/FAAN Criteria for Emergency Department Diagnosis of Anaphylaxis. J Allergy Clin Immunol Pract. 2016;4(6):1220–6.

For Peer Review

[30] Binder AM, Cherry-Brown D, Biggerstaff BJ, Jones ES, Amelio CL, Beard CB, et al. Clinical and laboratory features of patients diagnosed with alpha-gal syndrome—2010–2019. Allergy: European Journal of Allergy and Clinical Immunology. 2023;78(2):477–87.

[31] Sato T, Takio K, Kobata A, Greenwalt DE, Furukawa K. Site-Specific Glycosylation of Bovine Butyrophilin. The Journal of Biochemistry. 1995;117(1):147–57.

[32] Hvarregaard J, Andersen MH, Berglund L, Rasmussen JT, Petersen TE. Characterization of glycoprotein PAS-6/7 from membranes of bovine milk fat globules. Eur J Biochem. 1996;240(3):628–36.

For Peer Review

Figure legends

Figure 1. Investigation of the three bovine milk fractions: caseins (CAS), whey proteins (WP) and milk fat globule associated proteins (MFGP). Panel A: LDS page of MGFP, WP and CAS. Panel B: immunoblotting of MFGP, WP and CAS with the anti-α-Gal IgG antibody. Panel 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.

Figure 2. Investigation of α-gal bovine milk proteins. Panel 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 MGFPb de-glycosilated with PNGase for 3 hours (MFGPbDEG) and ON. (MFGPbDEGon). Panel B: immunoblotting of MFGP. MFGPb. MFGPbDEG. and MFGPbDEGon with anti-α-gal IgG. M: molecular weight; C+: thyroglobulin; CII: secondary antibody control.

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. Panel A: Immunoblotting of milk fat globule associated proteins (MFGP) with the sera of three patients (α2. α3. α5) immunoprecipitated with different concentrations of thyroglobulin (1. 3. 30. and 60 µg). Panel B: Immunoblotting of MFGP with the sera of three patients (α1. α2. 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.

For Peer Review

425 **Table 1.** Characterization of the patients.

ID PATIENT	SEX	AGE	CULPRIT FOOD	CLINICAL presentation	SYMPTOMS ONSET	TREATMENT	CO-FACTORS	TICK BITES	TOTAL SERUM IGE (KU/L)	ALPHA GAL IGEs (KUA/L)	TRYPTASE (ug/L)
Alpha1	M	37	Since 2020: always after eating red meat	Anaphylaxis: angioedema, urticaria, dyspnea	3-4 hours	Ebastine 10 mg at home Betamethasone 4mg at home	Physical exercise	Not known	117	3.08	5.50
Alpha2	F	74	9/2017: soup with egg pasta and veal kidney	Anaphylaxis: diffuse erythema, hypotension	3 hours	Chlorphenamine 10 mg (im) Betamethasone 4 mg (iv) Adrenaline 0.5 mg + 0.5 mg (im)	None	At least one tick bite in the past	117	0.37	6.19
			3/2018: tripe	Generalized urticaria	3 hours	Betamethasone 4mg at home Ebastine 10 mg at home					
Alpha3	F	69	9/2017: boiled meat, anchovies (parsley and garlic) and soup with beef broth	Anaphylaxis: generalized urticaria, palpebral oedema and vomiting	5 hours	Chlorphenamine 10 mg (im) Betamethasone 4 mg (iv)	None	Previous tick bites not known	702	86.50	6.30
			12/2018:	Severe	2 hours	O2-support					

For Peer Review

			offal and bread.	anaphylaxis: headache, loss of consciousness, hives, vomiting, respiratory failure		Chlorphenamine 10 mg (im) Methylprednisolone 80 mg (iv) Adrenaline 0.5 mg (im)					
			5/2019: 1 spoonful of veal broth.	Severe anaphylaxis: generalized urticaria, loss of consciousness	2 hours	Chlorphenamine 10 mg (im) Methylprednisolone 80 mg (iv) Adrenaline 0.5 mg (im)					
Alpha4	M	68	Since 2018: after eating offal	Urticaria	5-6 hours	Betamethasone 4mg at home Ebastine 10 mg at home	None	Many tick bites in the past	130	15.01	<1
			2022 stuffed pasta (meat)	Itching, urticaria	6 hours						
Alpha5	F	74	8/2017: lamb stew and pasta with red sauce	Anaphylaxis: generalized itching, urticaria, vomiting	3-4 hours	Chlorphenamine 10 mg (im) Methylprednisolone 100 mg (iv) Adrenaline 0.5 mg (im)	None	Previous tick bites not known	1383	2.54	8.13
			7/2018: pasta with tomato,	Generalized urticaria and	4 hours	Ebastine 10 mg at home					

For Peer Review

			lamb liver and lung (peppers and onions)	stomach-ache							
Alpha6	M	66	5/2018: carrots, "capocollo" and wine	Severe anaphylaxis: abdominal pain, nausea, vomiting, diarrhea, flushing, loss of consciousness	1 hour and half	O2-support Chlorphenamine 10 mg (im) Methylprednisolone 250 mg (iv) Adrenaline 0.5 mg (im)	Clonal mast cell disorder	Many tick bites in the past	309	11.60	10.20
			3 more similar episodes after ingestion of pork or offal.			Not available					
Alpha7	M	57	2010-2014: gummy bears	Recurrent urticaria	3-4 hours	Ebastine 10 mg at home	None	Many tick bites in the past	764	>100	14.60
			2016: red meat	Anaphylaxis: urticaria, hypotension, diarrhea	4-5 hours	Chlorphenamine 10 mg (im) Methylprednisolone 250 mg (iv)					
			2017: rabbit liver	Urticaria, angioedema	6 hours						
Alpha8	M	58	In 2018: always after eating meat	Anaphylaxis: angioedema, urticaria, abdominal	Not known	Chlorphenamine 10 mg (im) Methylpredn	None	Many tick bites in the past	162	31.50	9.12

For Peer Review

				pain, hypotension		isolone 250 mg (iv)					
Alpha9	F	26	Since 2015: after eating red meat	Recurrent urticaria and abdominal pain	6-7 hours	Betamethasone 4mg at home Ebastine 10 mg at home	None	At least one tick bite in the past	52.9	1.17	3.07
Alpha10	M	67	Since 2010: always after eating meat	Anaphylaxis: urticaria, dyspnea, peripheral edema)	5-6 hours	Chlorphenamine 10 mg (im) Methylprednisolone 250 mg (iv) Adrenaline 0.5 mg + 0.5 mg (im)	None	Not known	163	8.95	7.73
Healthy control	M	48	Since 2019: milk and dairy products, vegetarian	Diffuse itching and small wheals	2-12 hours	Cetirizine 10 mg at home	None	Not known	78	<0.10	3.80

F: female; M: male; im: intramuscular; iv: intravenous.

For Peer Review

Tab 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 protein, whey protein and casein fractions.

N° Band	Entry	Protein name	MW_{exp} / Mw_{theor} [Da]	Protein Score	N° of matching peptides	Protein coverage [%]
G1	P80457	Xanthine dehydrogenase	300000/142330	130.480	16	16
	Q8WNR8	Perilipin	300000/45251	52.718	8	27.9
	Q27960	Sodium-dependent phosphate transport protein 2B	300000/75825	52.365	5	6.6
	Q4GZT4	Broad substrate specificity ATP-binding cassette transporter ABCG2	300000/72724	43.296	7	15
	P18892	Butyrophilin subfamily 1 member A1	300000/59231	25.794	4	11
	A0A4W2HXW4	3-hydroxyacyl-[acyl-carrier-protein] dehydratase	300000/268170	12.606	2	1.1
G2	P80457	Xanthine dehydrogenase	130000/146790	317.140	30	30.1
	P18892	Butyrophilin subfamily 1 member A1	130000/59231	59.231	10	24.7
	A0A4W2I0L9	ATP-binding cassette sub-family G member 2	130000/67774	34.445	4	6
G3	G5E513	Ig-like domain-containing protein	80000/48107	31.553	9	23.2
	G5E5T5	Ig-like domain-containing protein	80000/55968	129.030	10	22.4
	A0A3Q1M193	Glycoprotein 2	80000/58465	260.530	8	17.3
	P18892	Butyrophilin subfamily 1 member A1	80000/59276	145.460	10	24.1
	C7FE01	Lactoferrin	80000/80278	55.906	8	12.8
	P80457	Xanthine dehydrogenase	80000/142330	32.807	5	3.8
G4	P81265	Polymeric immunoglobulin receptor	68000/82434	211.620	18	35.1
	A0A3Q1M193	Glycoprotein 2	68000/58465	92.215	10	23.8
	P18892	Butyrophilin subfamily 1 member A1	68000/59276	106.110	15	41.3
	P26201	Glycoprotein IIIb	68000/46055	91.212	6	12.9
	G5E513	Ig-like domain-containing protein	75000/48107	95.157	9	33.3
	A0A3Q1LWT4	Acyl-CoA synthetase long chain family member 1	68000/81442	79.564	10	18
	J7K1V4	Lactoferrin	68000/80278	75.774	12	18.6
	F1MHI1	Perilipin	80000/45281	53.926	7	25.5
	Q27960	Sodium-dependent phosphate transport protein 2B	68000/75825	32.389	5	9.7

For Peer Review

	Q95114	Milk fat globule-EGF factor 8 protein (Lactadherin)	68000/37465	32.227	4	17
	A0A3Q1MK38	Terpene cyclase/mutase family member	68000/74156	52.104	5	8.6
	P80457	Xanthine dehydrogenase	68000/142330	30.771	5	3.8
G5	P18892	Butyrophilin subfamily 1 member A1	60000/59276	252.710	19	31.4
	Q95114	Milk fat globule-EGF factor 8 protein (Lactadherin)	60000/43140	50.477	6	19
G6	Q95114	Milk fat globule-EGF factor 8 protein (Lactadherin)	51000/43140	198.570	22	57.2
	Q9TUM6	Perilipin-2	51000/49368	189.240	19	59.5
	P18892	Butyrophilin subfamily 1 member A1	51000/59276	31.353	5	13.3
G7	Q95114	Milk fat globule-EGF factor 8 protein (Lactadherin)	49000/43140	231.350	13	35.3
	Q8HZM7	Perilipin	49000/45281	55.801	4	18
G8	P02663	Alpha-S2-casein	34000/26018	41.439	6	18.9
	P18892	Butyrophilin subfamily 1 member A1	34000/59231	47.768	5	12.5
G9	B5B0D4	Major allergen beta-lactoglobulin	19000/19969	116.590	11	65.2
	Q5E9I6	ADP-ribosylation factor 3	19000/20601	47.494	7	45.9
	Q8HZM7	Perilipin	19000/45281	35.690	5	18.9
G10	P80457	Xanthine dehydrogenase	300000/142330	37.778	5	6.4
	A0A3Q1MGL5	SRCR domain-containing protein	300000/35988	16.338	2	23.1
	A0A4W2HXW4	3-hydroxyacyl-[acyl-carrier-protein] dehydratase	300000/268170	13.596	2	1.4
	Q27960	Sodium-dependent phosphate transport protein 2B	300000/75825	14.233	2	2.7
G11	P80457	Xanthine dehydrogenase	170000/142330	97.052	11	13
G12	P80457	Xanthine dehydrogenase	130000/146790	167.660	20	21.4
G13	P80457	Xanthine dehydrogenase	116000/14233	103.510	11	9.4
	Q27960	Sodium-dependent phosphate transport protein 2B	116000/75825	35.245	2	2.2
G14	G5E5T5	Immunoglobulin heavy constant mu	80000/56043	157.780	12	32.1
	F1MZQ4	Butyrophilin subfamily 1 member A1	80000/59231	65.440	7	15.8
G15	A0A4W2DWX4	Butyrophilin subfamily 1 member A1	60000/59245	94.962	13	25.9
G16	P0DOX5	Immunoglobulin gamma-1 heavy chain	53000/49328	97.277	10	31.2
	Q95114	Milk fat globule-EGF factor 8 protein (Lactadherin)	53000/37465	34.165	5	16.7

For Peer Review

G17	P01834	Immunoglobulin kappa constant	28000/11765	59.743	5	67.3
	A5PK49	Ig-like domain-containing protein	28000/24592	30.416	4	12.8
G18	A0A4W2I0L9	ATP-binding cassette sub-family G member 2	300000/67774	11.869	2	3.1
	P80457	Xanthine dehydrogenase	300000/146690	17.852	3	1.9
	Q27960	Sodium-dependent phosphate transport protein 2B	300000/75825	42.271	2	2.2
G19	P80457	Xanthine dehydrogenase	130000/146790	292.240	32	27
G20	G5E513	Immunoglobulin heavy constant mu	60000/56043	84.106	10	29.2
	P81265	Polymeric immunoglobulin receptor	60000/82434	65.441	9	17.6
	P18892	Butyrophilin subfamily 1 member A1	60000/59276	51.220	8	20
G21	F1MZQ4	Butyrophilin subfamily 1 member A1	57000/59231	63.366	7	17.5
G22	P18892	Butyrophilin subfamily 1 member A1	55000/59231	143.590	16	35.6
G23	Q9TUM6	Perilipin-2	48000/49368	83.058	8	28.9
	P18892	Butyrophilin subfamily 1 member A1	48000/59276	45.368	6	12.7
G24	Q95114	Milk fat globule-EGF factor 8 protein (Lactadherin)	44000/43140	137.390	16	39.2
	P18892	Butyrophilin subfamily 1 member A1	44000/59231	31.275	5	9.9
G25	P21163.2	Peptide-N(4)-(N-acetyl-beta-D-glucosaminyl)asparagine amidase PNGase F	40000/39032	227.360	16	52.5
	P18892	Butyrophilin subfamily 1 member A1	40000/59276	49.619	5	11.2
W1	P80457	Xanthine dehydrogenase	130000/146790	323.310	20	18.2
W2	A0A4W2CZN6	C3 complement	110000/190950	308.810	32	20.3
	A0A3Q1M3L6	Ig-like domain-containing protein	110000/40475	106.250	7	29.7
W3	C7FE01	Lactoferrin	75000/76274	323.310	45	66.5
	G5E513	Ig-like domain-containing protein	75000/48107	307.500	16	54.2
	G3X6N3	Serotransferrin	75000/77738	117.080	22	39.9
	P80025	Lactoperoxidase	75000/71350	187.390	22	39.9
	A0A3Q1M3L6	Ig-like domain-containing protein	75000/40475	44.510	4	19.3
	B3VTM3	Lactotransferrin	75000/78056	45.075	7	13
	A0A3Q1LNN7	Albumin	75000/68198	32.956	5	9.2
W4	P81265	Polymeric immunoglobulin receptor	68000/82434	134.530	11	25
	A0A4W2DZ09	Serotransferrin	68000/77738	133.090	15	33.5
	E1BMJ0	Serpin family G member 1	68000/51772	95.139	5	17.9
	A0A4W2CZN6	C3-beta-c	68000/190950	79.946	10	9

For Peer Review

	A0A3Q1M032	Ig-like domain-containing protein	68000/40475	92.036	4	16.800
	A0A4W2DDL5	Albumin	68000/68198	60.754	8	18.9
	A0A4W2GX34	Lactoperoxidase	68000/71350	33.890	5	9.2
W5	P02769	Albumin	60000/68198	323.310	41	64.7
	A0A4W2CZN6	C3 complement	60000/190950	244.450	28	22.2
	Q2KJF1	Alpha-1B-glycoprotein	60000/39566	75.560	9	36.2
W6	A0A3Q1M3L6	Ig-like domain-containing protein	50000/40475	148.910	10	52.9
	G3N0V0	Ig-like domain-containing protein	50000/35951	49.249	6	25.2
	Q9TTE1	Serpin A3-1	50000/46236	75.075	7	25.3
	A0A4W2HXY3	Serpin A3-1	50000/46815	33.062	5	17.9
	A0A140T8A9	Kappa-casein	50000/21237	30.867	3	23.7
	A0A3Q1NG86	Alpha-S1-casein	50000/23689	30.935	3	18
	P02754	Beta-lactoglobulin	50000/19883	32.047	3	22.5
	A0A4W2FAA0	Antithrombin-III	50000/52456	32.029	5	11.8
	P08037-2	Isoform Short of Beta-1.4-galactosyltransferase 1	50000/43483	79.095	2	5.1
W7	P02754	Beta-lactoglobulin	15000/19883	31.959	4	32.1
	P00711	Alpha-lactalbumin	15000/14156	144.240	3	24.4
	P80195	Glycosylation-dependent cell adhesion molecule 1	15000/17151	31.741	2	12.4
C1	P24627	Lactotransferrin	75000/78056	323.310	47	61.90
	P18892	Butyrophilin subfamily 1 member A1	75000/59276	41.743	3	12.20
	G5E513	Ig-like domain-containing protein	75000/48106	32.696	3	9.30
C2	P02662	Alpha-S1-casein	27000/23689	323.310	8	42.2
	P80195	Glycosylation-dependent cell adhesion molecule 1	27000/17151	93.318	2	12.4
	A0A140T8A9	Kappa-casein	27000/21237	190.770	4	30.5
	A0A452DHW7	Beta-casein	27000/29221	62.074	5	18.5
	P02754	Beta-lactoglobulin	27000/19883	61.784	5	37.1
	P02663	Alpha-S2-casein	27000/26018	62.470	3	13.5

430

431

For Peer Review

Tab 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 (D215VS)**
	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}N**S)
	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.

For Peer Review

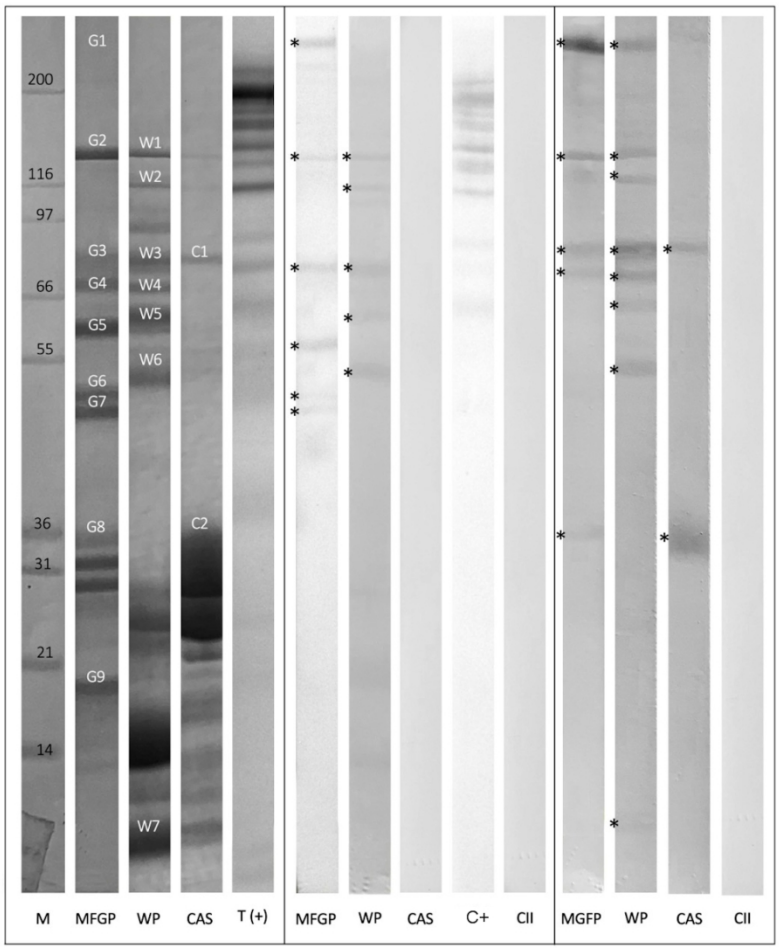


Figure 1. Investigation of the three bovine milk fractions: caseins (CAS), whey proteins (WP) and milk fat globule associated proteins (MFGP). Panel A: LDS page of MGFP, WP and CAS. Panel B: immunoblotting of MFGP, WP and CAS with the anti-α-Gal IgG antibody. Panel C: immunoblotting of MFGP, WP and CAS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

157x222mm (300 x 300 DPI)

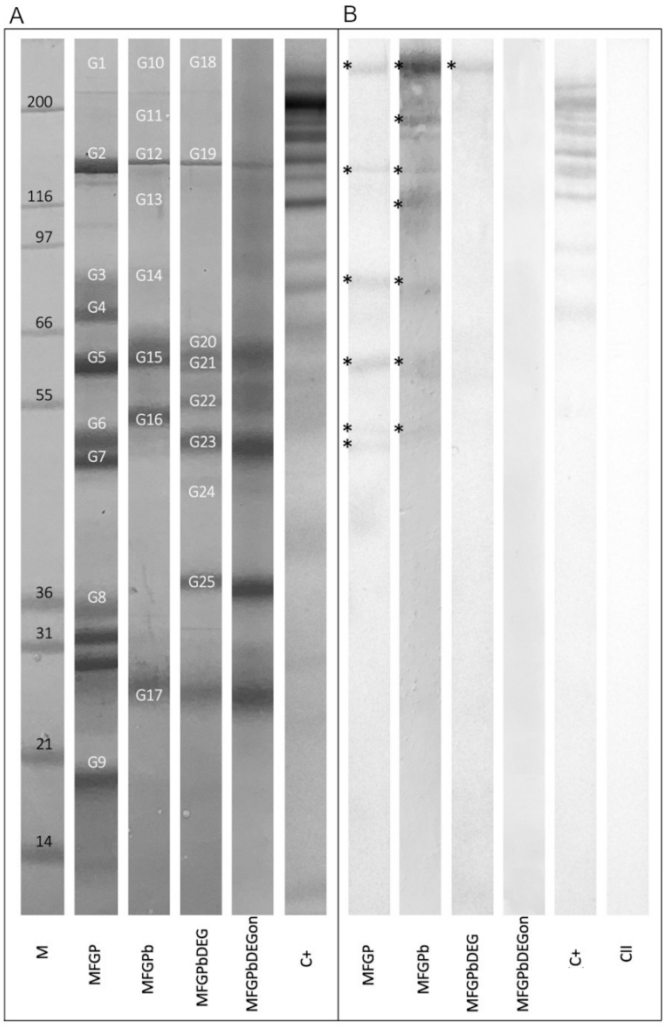


Figure 2. Investigation of α-gal bovine milk proteins. Panel A: LDS page of milk fat globule associated proteins (MFGP). MFGP enriched by means of incubation with beads bound with anti-α-gal IgG (MFGPb) and MGFPb de-glycosilated with PNGase for 3 hours (MFGPbDEG) and ON. (MFGPbDEGon). Panel B: immunoblotting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

157x222mm (300 x 300 DPI)

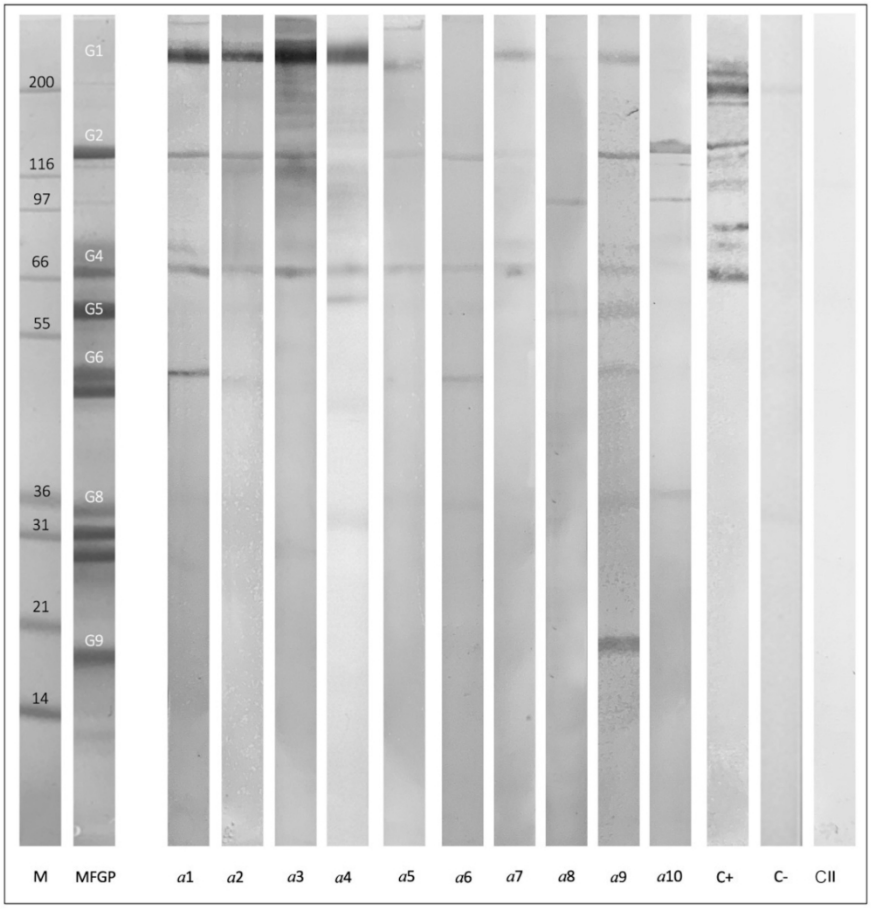


Figure 3. Recognition of milk fat globule associated proteins (MFGP) by α-gal syndrome (AGS) patients. Immunoblotting of MFGP with the sera of 10 AGS patients (from α1 to α10). M: molecular weight marker; C+: thyroglobulin. C-: patient not assuming meat. negative control CII: secondary antibody control.

175x222mm (300 x 300 DPI)

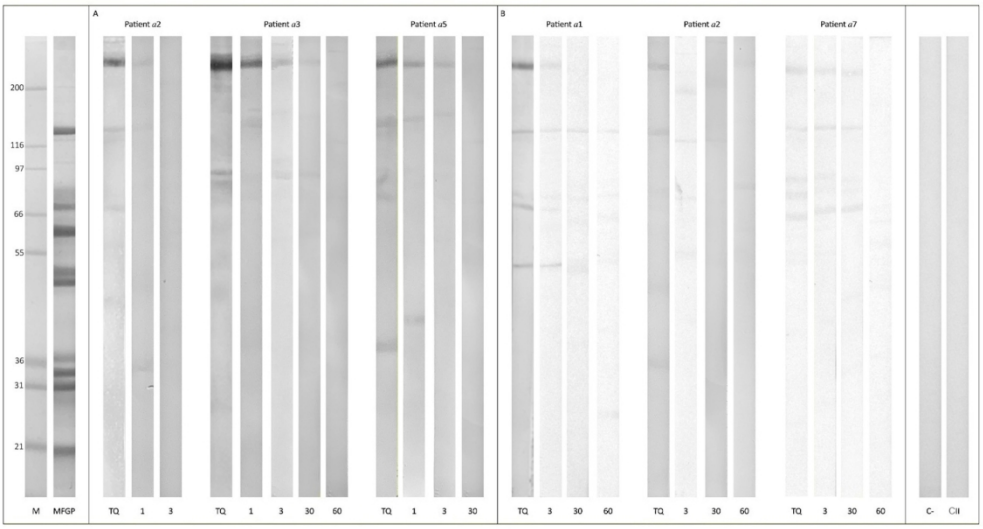


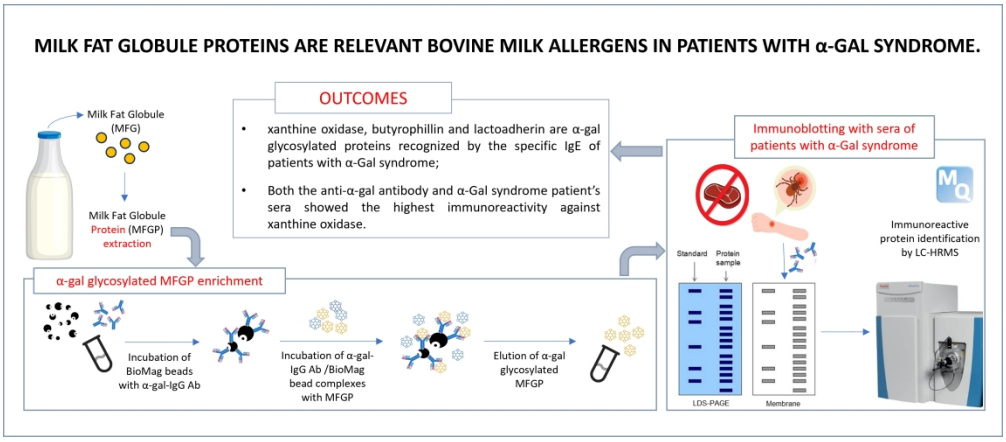
Figure 4. Immunoprecipitation experiments of α-gal syndrome (AGS) patient's sera. Panel A: Immunoblotting of milk fat globule associated proteins (MFGP) with the sera of three patients (α2. α3. α5) immunoprecipitated with different concentrations of thyroglobulin (1. 3. 30. and 60 μg). Panel B: Immunoblotting of MFGP with the sera of three patients (α1. α2. 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.

157x88mm (300 x 300 DPI)

Graphical Abstract Text

- 10-20% of patients affected by α-Gal syndrome (AGS) also react towards bovine milk.
- Alpha-gal glycosylated milk proteins were recognized by the IgE of AGS patients.
- Xanthine oxidase, butyrophillin and lactoadherin were found to be α-gal glycosylated.
- Xanthine oxidase is the milk protein most immunorecognized by the IgE of AGS patients.

For Peer Review



769x333mm (130 x 130 DPI)