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(Article begins on next page)

1 **Title**

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

3

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

27

28 **Key words:** alpha-gal syndrome, alpha-gal carbohydrate, food allergy, milk, xanthine oxidase.

29

30

31 **Abstract**

32 **Scope.** Alpha-gal syndrome (AGS) is a mammalian meat allergy associated with tick bites and specific
33 IgE to the oligosaccharide galactose- α -1,3-galactose (α -gal). Recent studies have shown that 10–
34 20% of AGS patients also react to the dairy proteins. Considering the already described role of the
35 meat lipid fraction in AGS manifestations, the aim of this work was to investigate whether the milk
36 fat globule proteins (MFGP) could be involved in AGS.

37 **Methods and results.** The MFGP were extracted and their recognition by the IgE of AGS patients
38 was proved through immunoblotting experiments.. The identification of the immunoreactive
39 proteins by LC-HRMS analysis allowed to demonstrate for the first time that butyrophillin,
40 lactadherin and xanthine oxidase are α -gal glycosylated. The role of xanthine oxidase seems to be
41 prevalent since it was highly immunerecognized by both the anti- α -gal antibody and AGS patient
42 sera.

43 **Conclusion.** The results obtained in this study provide novel insights in the characterization of α -Gal
44 carrying glycoproteins in bovine milk, supporting the possibility that milk, especially in its whole
45 form, may give reactions in AGS patients. Although additional factors are probably associated with
46 the clinical manifestations, the avoidance of milk and milk products should be considered in
47 individuals with AGS showing symptoms related to milk consumption.

48

49 1. Introduction

50 Alpha-gal syndrome (AGS) is a mammalian meat allergy associated with tick bites and specific IgE
51 antibodies to the oligosaccharide galactose- α -1,3-galactose (α -gal).^[1,2] Alpha-gal carbohydrate is
52 missing in humans and some primates, since the α -1,3-galactosyltransferase is expressed in an
53 enzymatically inactive form. It is instead present in most mammals, many pathogens such as
54 bacteria and parasites and in the salivary glands of several tick species, including the most prevalent
55 hard tick in Europe (*Ixodes ricinus*).^[3] Anti α -gal antibodies are the most abundant natural antibodies
56 in humans and some primates constituting up to 1% of the circulating antibodies. These antibodies
57 are mainly IgM and IgG class, but anti α -Gal IgEs can be also produced in some individuals suffering
58 from the red meat allergy.^[4] AGS symptoms vary from abdominal pain and diarrhea to urticaria and
59 anaphylaxis, the latter being experienced by nearly 50% of patients.^[5,6] AGS shows several exclusive
60 features that make it different from other food allergies: i) reactions are generally delayed,
61 appearing 3 to 6 hours after meat consumption; ii) IgE antibodies react to a carbohydrate moiety
62 rather than a protein epitope; iii) patients can develop AGS in late adulthood after a previous period
63 of meat tolerance.^[7] This atypical food allergy was first described in the southeastern regions of the
64 United States and in Australia, but it was also reported soon thereafter in Europe, Asia, Africa, and
65 Central America.^[8] More than 450000 cases have been described to date in the United States.^[9] The
66 frequency of positivity of specific IgE to α -Gal in Europe has been reported to be increasing in
67 northern countries (Denmark, Sweden, etc.), where it was first investigated,^[10,11] but also in Spain^[12]
68 and in the rural areas of northeast Italy.^[13] AGS is characterized by reactions to mammalian meat
69 and innards, including beef, pork, and lamb, as well as to food gelatins and some medications
70 (cetuximab, antivenom, gelatin-containing vaccines).^[14] Unlike common food allergies, the allergic
71 reactions may not occur at every exposure to the allergen. This variability depends on the amount
72 of allergen ingested and on the nature of the biologic macromolecules within the α -gal-containing

73 food. Lipid-rich mammalian meats are associated with more consistent and severe reactions.^[15]
74 Lipid-bound α -Gal appears to be able to cross the intestinal monolayer and to trigger an allergic
75 reaction, thus suggesting that not only glycoproteins but also glycolipids should be investigated as
76 potential allergenic molecules.^[16] Chakrapani et al.,^[17] have recently confirmed the involvement of
77 glycolipids in the activation of AGS patient basophils, even if the major role played by glycoproteins,
78 particularly those from pork kidneys and beef extracts, is already well established. Glycolipids
79 extracted from these food matrices have shown a lower basophil activation capacity than their
80 respective protein extracts.^[18]

81 Not only red meat but also bovine milk contains α -Gal-epitopes, although in smaller amounts.^[19]
82 Some recent studies,^[7,20,21] including one considering a large cohort of 2,500 AGS patients in the
83 USA,^[22] have demonstrated that 10–20% of AGS patients also react to milk. The most reported
84 symptoms in AGS patients following bovine milk ingestion are abdominal pain and urticaria with a
85 delayed onset of the symptoms.^[23] Unlike meat, where α -Gal-bearing proteins have long been
86 extensively studied, sources containing α -Gal epitopes in dairy products have only recently been
87 investigated^[24], by a modified inhibition RIA (Radio Immuno Assay), were able to detect α -Gal
88 proteins in heavy milk cream but no detectable α -Gal was found in skimmed cow milk or 1%, 2%
89 milk fat. Perusko et al.^[25] demonstrated that bovine γ -globulin (BGG), lactoferrin (LF), and
90 lactoperoxidase (LPO) are α -Gal carrying proteins that have been recognized by the IgEs of AGS
91 patients and which are able to activate the basophils of patients. More recently, the same α -Gal
92 glycosylated proteins were found in sheep milk by German-Sanchez et al.^[26]

93 Milk lipid fraction consists of lipid globules surrounded by membrane. The MFG are assembled and
94 secreted by the epithelial cells of the mammary gland and consist of a complex mixture of proteins,
95 enzymes, neutral lipids, and phospholipids enriched with glycoproteins leaving the mammary cell
96 by exocytosis.^[27] During the last years MFG proteins (MFGP) have been reported to have impact on

97 several cellular processes such as inflammation, differentiation, antimicrobial and antiadhesive
98 properties, and proliferation of intestinal epithelial cells.^[28] MFGP represent 1–4% of the total milk
99 proteins. The major MFGP in bovine milk are adipophilin (ADPH), butyrophilin (BTN), mucins (MUC1,
100 MUC 4, MUC15), xanthine dehydrogenase/oxidase (XDH/XO), CD36, lactadherin (LA), periodic acid
101 Schiff III (PAS III) and fatty acid-binding protein (FABP).^[29] Proteomics has been employed in the
102 identification and characterization of MFGP.^[30,31] In term of glycoproteomic studies, MFGM N-
103 glycoproteins, including butyrophilin, lactadherin, mucins, integrins, and immunoglobulins, have
104 been successfully studied.^[28,32] Less is known concerning the galactose- α -1,3-galactose determinant
105 on bovine MFGP.

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

110

111 **2. Experimental Section**

112 **2.1 Characterization of the patients**

113 This observational study was carried out on 10 adults Italian AGS patients at the Allergy and Clinical
114 Immunology University Clinic in Turin (AO Ordine Mauriziano di Torino). Details about the
115 characterization of patients enrolled in the study are available in the Online Repository Material and
116 Method and in Table S1.

117

118 **2.2 Chemicals**

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

120 **2.3 Milk fat globule membrane associated protein extraction**

121 The MFGP was extracted according to Barello et al.^[34] The milk protein fractions were extracted
122 from 60 mL of whole fresh cow's milk centrifugated at 5.000xg for 30 minutes at 6°C to remove
123 somatic cells and impurities. Caseins (CAS), whey proteins (WP), and fat globules were obtained by
124 means of high-speed centrifugation (189.000xg for 70 minutes at 6°C) and stored at -20°C.

125 In order to extract MFGP, 300 µl of extraction buffer (5mM Tris-HCl pH 8.8; 6,5M urea; 2,2 M
126 thiourea; 1% w/v ASB-14)^[35] was added to the milk fat globule pad (200 µl). The sample was
127 incubated under agitation at room temperature (RT) for 1h and centrifugated at 21.000xg for 30
128 sec. After removing the floating cream layer, the supernatant containing the MFGP was collected
129 and precipitated with methanol and chloroform, as described by Wessel and Flügge,^[36] to remove
130 any salts or detergent residues. The protein pellet was quantified by means of 2-D QuantKit.

131 **2.4 Glycosylated milk fat globule membrane associated protein (MFGP) enrichment**

132 Sixty µl of BioMag Goat Anti-Human IgG beads (5,2mg/ml) (BioMag beads) were washed twice with
133 500 µl of PBS. The washed BioMag beads were blocked twice with TBS with 0,3% of Tween 20
134 (blocking solution) for 15 minutes under agitation at 4°C. After removing the BS, the BioMag beads
135 were incubated with 1:1 of human IgG1 anti α -gal-epitope antibody (α -gal-IgG Ab) or 6 hours under
136 rotation at 4°C. The α -gal-IgG Ab /BioMag bead complexes were collected by means of a magnetic
137 bar and were washed twice with 500 µl of PBS. Sixty µg of MFGP were added to the α -gal-IgG Ab
138 /BioMag bead complex and incubated overnight (O.N.) at 4°C. The α -gal-IgG antibody /BioMag bead
139 / MFGP complexes were then collected again and washed twice with 500 µl of PBS. The MFGP and
140 α -gal-IgG antibodies were released from the BioMag beads by incubating them with the elution
141 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
142 released from the beads were then used in the subsequent experiments.

143 **2.5 Milk fat globule membrane associated protein N-de-glycosylation**

144 Enzymatic removal of the N-linked glycans was performed using PNGase F, a glycan-Asn-amidase
145 that specifically cleaves the innermost GlcNAc of all N-linked oligosaccharides, unless they carry $\alpha(1-$
146 $3)$ core-bound fucose residues.^[37] The experiment was carried out under denaturing conditions: 40g
147 of proteins were resuspended in a modified Laemmli buffer (60mM Tris-HCl pH 6.8, 0,25% SDS, 10%
148 glycerol) and 1ul of 1M DTT was added. The sample was incubated at 95°C for 5 min. After cooling,
149 2 μ l of 10% NP-40 and a quantity of PNGase F (10U/ μ g) were added, in a 1:1 enzyme/substrate ratio,
150 to the sample and incubated at 37 °C for 3 hours and overnight (ON) under slight shaking.

151 **2.6 Protein separation and LC-HRMS analysis**

152 The LDS-PAGE separation and LC-HRMS analysis were performed according to Cirrincione et al.^[38]
153 Protein separation was performed using Lithium dodecyl sulfate-PAGE (LDS-PAGE) with precast gels
154 (NuPAGE 4–12% Bis–Tris gels) and MES Running buffer in an XCell SureLock Mini-Cell System
155 (Invitrogen), according to the manufacturer's instructions. Each sample was diluted in a NuPage LDS
156 Sample Buffer, under reducing conditions (1% of NuPage sample reducing agent) and loaded in an
157 equal amount (5 μ g). LMW standards were run as the molecular weight reference. Gels were stained
158 with Colloidal Coomassie Blue^[39] and scanned with a ChemiDoc MP System densitometer (Bio-Rad)
159 at 600 dpi of resolution.

160 The selected LDS PAGE bands were cut and reduced in 10 mM DTT/50 mM NH_4HCO_3 for 45 min at
161 56 °C and subsequently alkylated in 55 mM IAA/50 mM NH_4HCO_3 for 30 min at room temperature
162 in the dark. They were then de-stained with ACN 50%/50 mM NH_4HCO_3 , pure ACN and, again with
163 ACN 50%/50 mM NH_4HCO_3 . The samples were dried in the 5301 Eppendorf Concentrator
164 (Eppendorf, Hamburg, Germany) and digested with 7 μ l of modified porcine proteomic grade trypsin
165 (75 ng/ μ l) in 25 mM NH_4HCO_3 /10% ACN, at 37 °C, O.N., under shaking. One μ l of 5% FA was added

166 to stop the enzymatic protein digestion. The Orbitrap Q Exactive Plus, coupled to a UHPLC binary
167 pump system (Vanquish Thermo Fisher Scientific, Waltham, Massachusetts, USA), was used to
168 perform protein identification. The stationary phase was a BioBasic™ C18 HPLC Column (1 × 150
169 mm, 5 μm; Thermo Scientific). The mobile phases were 0.1% (v/v) FA in MilliQ water (A) and 0.1%
170 (v/v) FA in ACN (B), and they were eluted at a flowrate of 50.0 μL/min with increasing concentrations
171 of solvent B, from 5% to 70%, over 50 min and with 80% for 5 min. The oven temperature was set
172 at 55 °C. The autosampler was set at 6 °C. The injection volume was 4.0 μL. Mass spectra were
173 acquired in Full MS-ddMS2 mode. The instrument was set up so that Full MS spectra were acquired
174 in an m/z scan range of 150-1800, the resolution was set at 70,000, the maximum IT was 200 ms,
175 the AGC target was 5x10⁵, and the charge exclusion was unassigned. Up to 12 of the most intense
176 ions in MS1 were selected for fragmentation in the MS/MS mode. The fragmentation spectra
177 resolution was set at 17,500 for the MS/MS spectra, with a dynamic exclusion of 20 s and an isolation
178 window of 2.0 m/z, while the normalized collision energy was set at 28, the maximum IT at 200 ms
179 and the AGC target at 2x10⁴.

180 **2.7 Protein identification strategy**

181 All the Data Dependent Analysis (DDA) files were searched using MaxQuant (<https://maxquant.org>)
182 v. 2.0.3.0 against the UniProt *Bos taurus* database (reviewed and unreviewed). The search was
183 performed using a list of contaminants devoid of bovine proteins, because they were our target.
184 The search parameters were set as follow: S-carbamidomethyl derivate on cysteine as a fixed
185 modification, oxidation on methionine, Acetyl (N-term) as variable modifications and two missed
186 cleavage sites for trypsin digestion. The possibility of Asn becoming Asp was added as a variable
187 modification for bands derived from enzymatic de-glycosylation. The MS/MS fragment mass
188 tolerance was set at 20 ppm. A minimum of 3 peptides, an FDR of 0.01% for both the protein and
189 peptides, and a score of 20 for unmodified and modified peptides were set for the protein

190 identification. Only proteins identified with a score >35 were listed in the tables, with the exception
191 of the identification performed on unstained bands cut in the upper part of the gels where a score
192 of >10 was allowed.

193 **2.8 Whey and milk fat globule membrane associated protein immunoblotting**

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

206 **2.9 Immunoprecipitation of the AGS patient sera**

207 Immunoprecipitation experiments were performed with two glycosylated proteins: bovine
208 thyroglobulin and the bovine xanthine oxidase from Sigma-Aldrich. The sera of three AGS patient
209 (α 2, α 3, α 5) were incubated for 1h at room temperature with four amounts of thyroglobulin (1, 3,
210 30, 60 μg) and other three patients (α 1, α 2, α 7) were incubated at the same conditions with three
211 amounts of xanthine oxidase (3, 30, 60 μg). Nitrocellulose membranes containing electro-
212 transferred MFGP were blocked with the blocking solution for 30 min and then incubated overnight

213 at 4°C with the immunoprecipitated sera. The immunoblotting procedure was then performed as
214 previously explained in Section 2.7.

215

216 **3. Results**

217 **3.1 Study population**

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

221 **3.1.1 Clinical presentation of AGS**

222 All the AGS patients reported at least one delayed reaction (average 3.40 ± 1.58 events/person) with
223 a mean onset time of 4.1 hours after eating red meat, innards, or meat-related food (Table 1, Table
224 S1). None of the patients was allergic to cow's milk. The most common culprit food was pork meat.
225 Urticaria was the most common clinical manifestation (100%), followed by gastrointestinal
226 symptoms (vomiting, diarrhea, and abdominal pain) (60%), hypotension (50%), angioedema (50%)
227 and dyspnea (30%). Nine patients (90%) had at least one episode of anaphylaxis, diagnosed
228 according to NIAID/FAAN criteria.^[40] No cofactor of anaphylaxis, including ethanol, or nonsteroidal
229 anti-inflammatory drug consumption was identified, apart from one patient who reported
230 anaphylaxis after red meat ingestion and physical exercise. None of our patients had previously
231 been treated with cetuximab. Eight patients (80%) reported one or multiple tick bites before AGS.
232 All the patients were positive to α -gal specific IgE ($26,08 \pm 35.87$ KUA/l) with a mean serum total IgE
233 of 389.99 ± 429.94 KU/l. Tryptase resulted normal in all the patients, with a mean value of $7,18 \pm 3.78$
234 $\mu\text{g/l}$).

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

238

239 **3.2 The Anti- α -gal antibody recognizes whey and milk fat globule proteins**

240 The milk fat globule proteins (MFGP), whey proteins (WP), and caseins (CAS) were separated by
241 means of LDS page followed by immunoblotting analysis with anti- α -Gal IgG and a pool of sera from
242 10 AGS patients (Fig 1). Both the MFGP and WP extracts showed immunoreactive bands for anti- α -
243 Gal IgG: G1, G2, G3, G5, G6, G7 and W1, W2, W3, W5, W6, respectively (Fig 1, panel B). LC-HRMS
244 analysis (Table 2, Table S2) allowed LF and LPO to be identified in band W3, and several Ig-like
245 domain containing proteins were identified in bands W2, W3, W5, W6. Xanthine Oxidase (XO) was
246 identified in W1 and in several reactive bands of MFGP (G1, G2 and G3), while the other reactive
247 bands (G3, G5, G6 and G7) mainly contained butyrophilin (BT) and lactadherin (LA).

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

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

257 **3.3 Xanthine oxidase, butyrophilin and lactadherin are α -Gal-glycosylated proteins**

258 In order to enrich the sample in α -Gal-glycosylated proteins, we isolated glycosylated MFGP using
259 BioMag Goat Anti-Human IgG beads conjugated with the anti- α -gal IgG system. After the
260 enrichment, the proteins were separated by means of LDS PAGE (Fig. 2, panel A, lane MFGPb). The
261 thus isolated MFGP resulted to be high molecular weight proteins and as expected, they were
262 recognized by the anti- α -gal IgG. However, the situation was different for bands G16, G17 and G25,
263 as they contained heavy and light anti- α -gal IgG chains partially released from the beads during
264 protein elution, and PNGase F, the enzyme used for de-glycosylation. In addition to the heavy anti-
265 α -gal IgG chain, LA was identified in band G16, which is probably responsible for the corresponding
266 immunoreactivity, while the other two bands did not result to be immunoreactive. When the α -Gal-
267 enriched protein sample was de-glycosylated with PNGase F, the anti- α -gal IgG did not recognize any
268 band, except for a slight recognition of G18 where XO was present (Fig. 2, Panel A, lane MFGPbDEG).
269 This reactivity completely disappeared only after a more exhaustive overnight PNGase F de-
270 glycosylation (Fig. 2, panel A, lane MFGPbDEGon). The analysis of the bands containing the N-de-
271 glycosylated proteins that lost reactivity revealed which asparagine could carry the α -gal moiety (Fig.
272 2 panel A and B, lane MFGPbDEG). The presence of new tryptic peptides with aspartic acid instead
273 of the original asparagine was considered as proof of the presence of a glycosylation site carrying
274 the α -gal sugar chain on the peptide before digestion. The LC-HRMS study of the G22 band showed
275 a BT peptide with Asn₂₁₅ modified to Asp₂₁₅ after the de-glycosylation protocol. The same was
276 observed in band G24, where LA showed an Asn₂₂₇ modified to Asp₂₂₇. All these results are
277 summarized in Table 3.

278

279 **3.4 The AGS patients' IgE antibodies recognize Xanthine Oxidase and Butyrophilin**

280 The MFGP sample was also incubated with the serum of each single patient (Fig. 3). As in previous
281 experiments, the most recognized bands were G1 (recognized by 7/10 patients), G2 (8/10 patients)

282 and G4 (8/10 patients), which mainly contain XO and BP. Bands G5 and G8, which showed a reduced
283 recognition rate, were recognized by 2/10 patients, while G6 was recognized by 3/10 patients and
284 G9 by only 1 patient. Once again, these bands mainly contained XO, but also LA and β -lactoglobulin.

285 In order to verify that the patient immunorecognition was addressed to α -gal epitopes,
286 immunoprecipitation of three patients' sera (α 2, α 3, α 5) was performed with four concentrations
287 of bovine thyroglobulin (1, 3, 30, 60 μ g) (Fig. 4, Panel A). Only patient α 3 needed 60 μ g of
288 thyroglobulin to completely inhibit the immunorecognition. Instead, for the other two patients, 3
289 or 30 μ g was sufficient. The same experiment was performed with bovine XO (patients α 1, α 2, α 7)
290 (Fig. 4, Panel B). In this case, 60 μ g of XO was needed to immunoprecipitate the patients' sera.
291 Patient α 2, who was tested in both inhibition experiments, needed 60 μ g of XO and only 3 μ g of
292 thyroglobulin.

293

294 **4. Discussion**

295 Patients with AGS have been known to report allergic manifestations associated with the ingestion
296 of dairy products, due to the presence of α -Gal carrying proteins, which have recently been
297 identified in bovine milk whey.^[7,19,22,25] In order to prove that these milk-induced allergic reactions
298 are due to IgE recognizing α -Gal, it is necessary to exclude other more common causes of reactions
299 to milk, including lactose intolerance and cow's milk allergy.^[41] In the present work, we have found
300 that milk fat globule associated proteins contain α -Gal epitopes recognized by the specific IgE of
301 patients with AGS. Specifically, we have demonstrated, for the first time, that BT, LA, and XO
302 contained in milk fat globules are α -gal glycosylated. The pool of patients' sera also immune-
303 recognized milk LF, LPO, and IgG-like proteins, as expected.

304 The α -gal-glycosylation of BT, LA, and XO was confirmed by means of immunoblotting experiment,
305 since immunorecognition by the anti- α -gal IgG and by AGS patients' sera was lost after de-
306 glycosylation. The LC-HRMS approach showed that new tryptic peptides containing Asp instead of
307 Asn were generated after enzymatic de-glycosylation giving reason for possible α -gal-glycosylation
308 sites on these proteins. Although the glycosylation sites of BT and LA had previously been identified
309 by Sato et al.^[42] and by Hvarregaard et al.^[43] we have identified, for the first time, the glycosylation
310 site of XO (Asn₇₀₄ modified to Asp₇₀₄).

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

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

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

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

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

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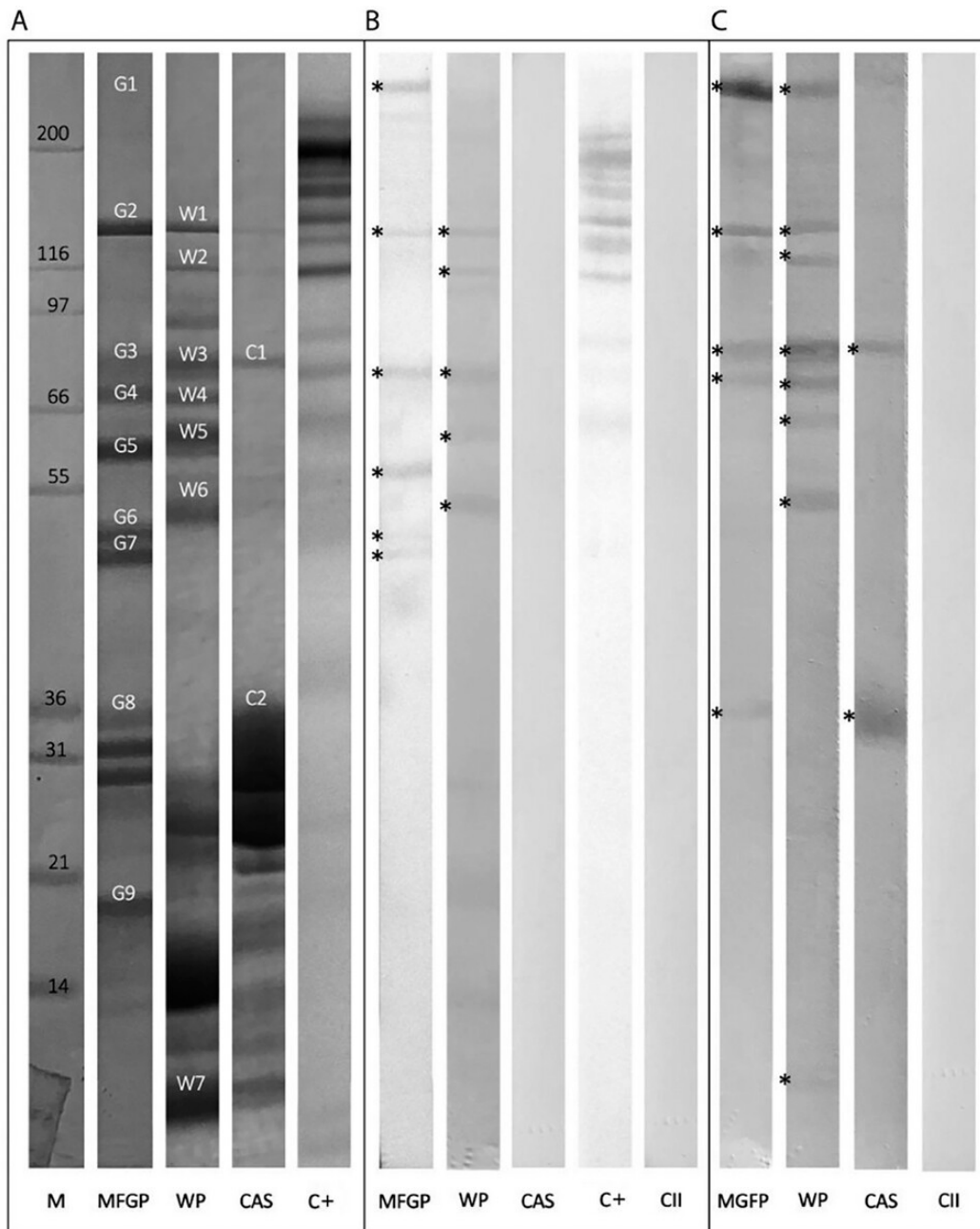
335 Bibliography

- 336 1. Commins SP, Satinover SM, Hosen J, Mozena J, Borish L, Lewis BD, et al. Delayed anaphylaxis,
337 angioedema, or urticaria after consumption of red meat in patients with IgE antibodies
338 specific for galactose- α -1,3-galactose. *Journal of Allergy and Clinical Immunology*.
339 2009;123(2):426-433.e2.
- 340 2. Fischer J, Yazdi AS, Biedermann T. Clinical spectrum of α -Gal syndrome: From immediate-type
341 to delayed immediate-type reactions to mammalian innards and meat. *Allergo J Int*.
342 2016;25(2):55–62.
- 343 3. Apostolovic D, Mihailovic J, Commins SP, Wijnveld M, Kazimirova M, Starkhammar M, et al.
344 Allergenomics of the tick *Ixodes ricinus* reveals important α -Gal-carrying IgE-binding proteins
345 in red meat allergy. *Allergy: European Journal of Allergy and Clinical Immunology*.
346 2020;75(1):217–20.
- 347 4. Boussamet L, Montassier E, Soullillou JP, Berthelot L. Anti α 1-3Gal antibodies and Gal content
348 in gut microbiota in immune disorders and multiple sclerosis. Vol. 235, *Clinical Immunology*.
349 Academic Press Inc.; 2022.
- 350 5. Kennedy JL, Stallings AP, Platts-Mills TAE, Oliveira WM, Workman L, James HR, et al.
351 Galactose- α -1, 3-galactose and delayed anaphylaxis, angioedema, and urticaria in children.
352 *Pediatrics*. 2013;131(5).
- 353 6. Young I, Prematunge C, Pussegoda K, Corrin T, Waddell L. Tick exposures and alpha-gal
354 syndrome: A systematic review of the evidence. *Ticks Tick Borne Dis*. 2021;12(3):101674.
- 355 7. Kiewiet MBG, Apostolovic D, Starkhammar M, Grundström J, Hamsten C, van Hage M. Clinical
356 and Serological Characterization of the α -Gal Syndrome—Importance of Atopy for Symptom
357 Severity in a European Cohort. *Journal of Allergy and Clinical Immunology: In Practice*.
358 2020;8(6):2027-2034.e2.
- 359 8. van Nunen S. Galactose-Alpha-1,3-Galactose, Mammalian Meat and Anaphylaxis: A World-
360 Wide Phenomenon? *Curr Treat Options Allergy*. 2014;1(3):262–77.
- 361 9. Thompson JM, Carpenter A, Gilbert ;, Kersh J, Wachs ; Tyler, Commins SP, et al. Morbidity and
362 Mortality Weekly Report Geographic Distribution of Suspected Alpha-gal Syndrome Cases-
363 United States [Internet]. Vol. 72, Centers for Disease Control and Prevention | MMWR. 2023.
364 Available from: <https://ndc.services.cdc.gov/case-definitions/alpha-gal-syndrome-ags/>
- 365 10. Apostolovic D, Krstic M, Mihailovic J, Starkhammar M, Cirkovic Velickovic T, Hamsten C, et al.
366 Peptidomics of an in vitro digested α -Gal carrying protein revealed IgE-reactive peptides. *Sci*
367 *Rep*. 2017;7(1):1–10.
- 368 11. Gonzalez-Quintela A, Dam Laursen AS, Vidal C, Skaaby T, Gude F, Linneberg A. IgE antibodies
369 to alpha-gal in the general adult population: Relationship with tick bites, atopy, and cat
370 ownership. *Clinical and Experimental Allergy*. 2014;44(8):1061–8.
- 371 12. Mateo-Borrega MB, Garcia B, Larramendi CH, Azofra J, González-Mancebo E, Alvarado MI, et
372 al. Ige-mediated sensitization to galactose- α -1,3-galactose (α -gal) in urticaria and anaphylaxis

- 373 in spain: Geographical variations and risk factors. *J Investig Allergol Clin Immunol.*
374 2019;29(6):436–43.
- 375 13. Villalta D, Cecchi L, Farsi A, Chiarini F, Minale P, Voltolini S, et al. Galactose- α -1,3-galactose
376 syndrome: An Italian survey. *Eur Ann Allergy Clin Immunol.* 2017;49(6):263–9.
- 377 14. Platts-Mills TAE, Commins SP, Biedermann T, van Hage M, Levin M, Beck LA, et al. On the
378 cause and consequences of IgE to galactose- α -1,3-galactose: A report from the National
379 Institute of Allergy and Infectious Diseases Workshop on Understanding IgE-Mediated
380 Mammalian Meat Allergy. *Journal of Allergy and Clinical Immunology.* 2020;145(4):1061–71.
- 381 15. Iweala OI, Choudhary SK, Addison CT, Batty CJ, Kapita CM, Amelio C, et al. Glycolipid-
382 mediated basophil activation in alpha-gal allergy. *Journal of Allergy and Clinical Immunology.*
383 2020;146(2):450–2.
- 384 16. Román-Carrasco P, Lieder B, Somoza V, Ponce M, Szépfalusi Z, Martin D, et al. Only α -Gal
385 bound to lipids, but not to proteins, is transported across enterocytes as an IgE-reactive
386 molecule that can induce effector cell activation. *Allergy: European Journal of Allergy and
387 Clinical Immunology.* 2019;74(10):1956–68.
- 388 17. Chakrapani N, Fischer J, Swiontek K, Codreanu-Morel F, Hannachi F, Morisset M, et al. α -Gal
389 present on both glycolipids and glycoproteins contributes to immune response in meat-
390 allergic patients. *Journal of Allergy and Clinical Immunology.* 2022;150(2):396-405.e11.
- 391 18. Carson AS, Gardner A, Iweala OI. Where’s the Beef? Understanding Allergic Responses to Red
392 Meat in Alpha-Gal Syndrome. *The Journal of Immunology.* 2022;208(2):267–77.
- 393 19. Commins SP. Invited Commentary: Alpha-Gal Allergy: Tip of the Iceberg to a Pivotal Immune
394 Response. *Curr Allergy Asthma Rep.* 2016;16(9):1–3.
- 395 20. Armstrong P, Binder A, Amelio C, Kersh G, Biggerstaff B, Beard C, et al. Descriptive
396 Epidemiology of Patients Diagnosed with Alpha-gal Allergy — 2010–2019. *Journal of Allergy
397 and Clinical Immunology.* 2020;145(2):AB145.
- 398 21. Wilson JM, Schuyler AJ, Workman L, Gupta M, James HR, Posthumus J, et al. Investigation
399 into the α -Gal Syndrome: Characteristics of 261 Children and Adults Reporting Red Meat
400 Allergy. *Journal of Allergy and Clinical Immunology: In Practice.* 2019;7(7):2348-2358.e4.
- 401 22. Commins SP. Diagnosis & management of alpha-gal syndrome: lessons from 2,500 patients.
402 *Expert Rev Clin Immunol.* 2020;16(7):667–77.
- 403 23. Choi G -S., Kim J -H., Shin Y -S., Nahm D -H., Park H -S. Food allergy to meat and milk in adults.
404 *Allergy.* 2010 Aug;65(8):1065–7.
- 405 24. Mullins RJ, James H, Platts-Mills TAE, Commins S. Relationship between red meat allergy and
406 sensitization to gelatin and galactose- α -1,3-galactose. *Journal of Allergy and Clinical
407 Immunology.* 2012;129(5).
- 408 25. Perusko M, Apostolovic D, Kiewiet MBG, Grundström J, Hamsten C, Starkhammar M, et al.
409 Bovine γ -globulin, lactoferrin, and lactoperoxidase are relevant bovine milk allergens in

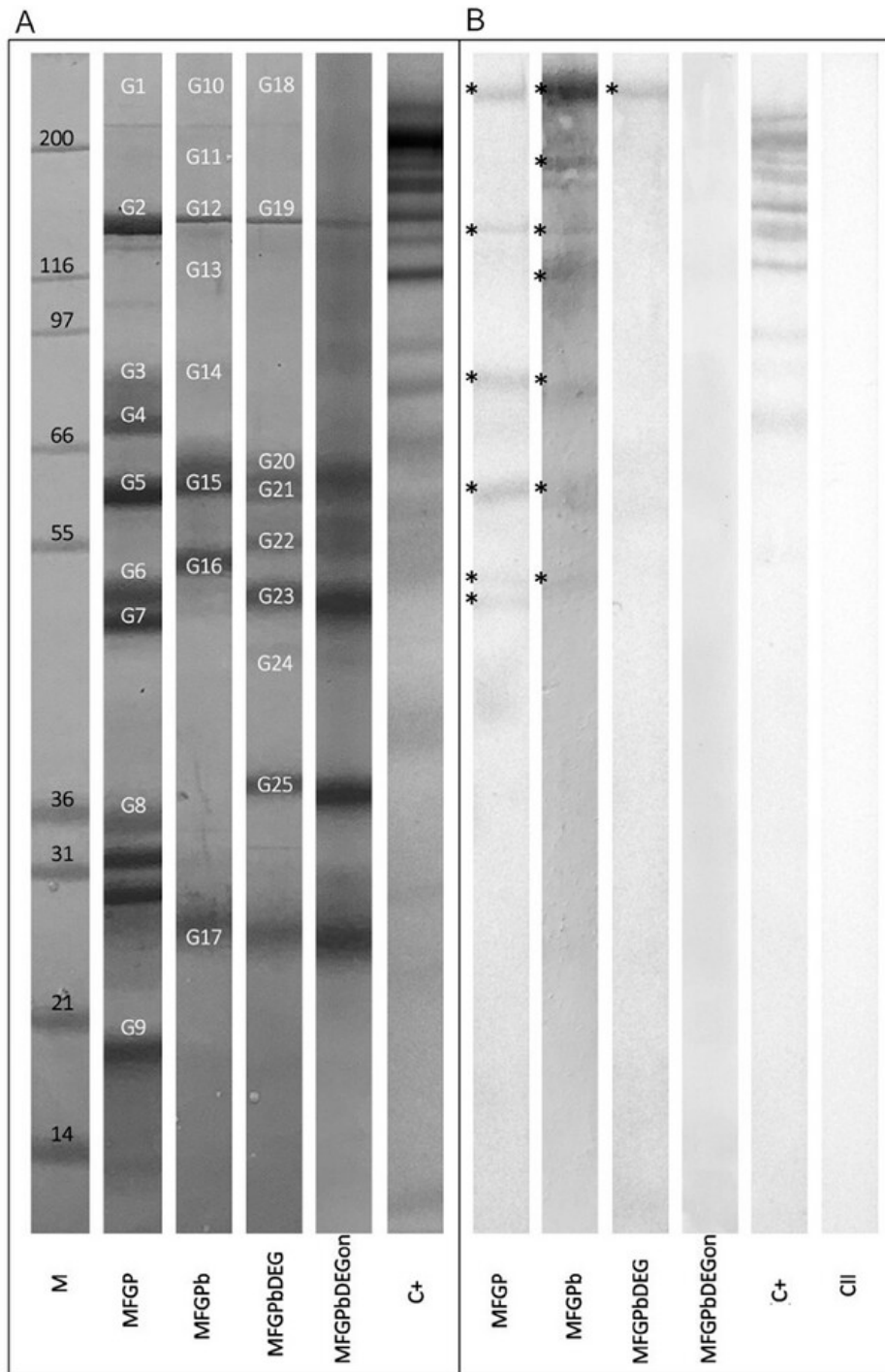
- 410 patients with α -Gal syndrome. *Allergy: European Journal of Allergy and Clinical Immunology*.
411 2021;76(12):3766–75.
- 412 26. German-Sanchez A, Alonso-Llamazares A, Latorre-Ibañez M, Bartolome-Zavala B, Antepará-
413 Ercoreca I. Sheep cheese allergy in Alpha-gal Syndrome. *J Investig Allergol Clin Immunol*.
414 2023;33(6):1–7.
- 415 27. Brink LR, Lönnerdal B. The role of milk fat globule membranes in behavior and cognitive
416 function using a suckling rat pup supplementation model. *Journal of Nutritional Biochemistry*.
417 2018 Aug 1;58:131–7.
- 418 28. Guan B, Zhang Z, Chai Y, Amantai X, Chen X, Cao X, et al. N-glycosylation of milk proteins: A
419 review spanning 2010–2022. Vol. 128, *Trends in Food Science and Technology*. Elsevier Ltd;
420 2022. p. 1–21.
- 421 29. Manoni M, Cattaneo D, Mazzoleni S, Giromini C, Baldi A, Pinotti L. Milk Fat Globule Membrane
422 Proteome and Micronutrients in the Milk Lipid Fraction: Insights into Milk Bioactive
423 Compounds. Vol. 2, *Dairy*. MDPI; 2021. p. 202–17.
- 424 30. Spertino S, Cipriani V, De Angelis C, Giuffrida MG, Marsano F, Cavaletto M. Proteome profile
425 and biological activity of caprine, bovine and human milk fat globules. *Mol Biosyst*. 2012
426 Mar;8(4):967–74.
- 427 31. Reinhardt TA, Lippolis JD. Bovine milk fat globule membrane proteome. *Journal of Dairy*
428 *Research*. 2006 Nov;73(4):406–16.
- 429 32. Yang Y, Zheng N, Wang W, Zhao X, Zhang Y, Han R, et al. N-glycosylation proteomic
430 characterization and cross-species comparison of milk fat globule membrane proteins from
431 mammals. *Proteomics*. 2016 Nov 1;16(21):2792–800.
- 432 33. Wilson JM, Erickson L, Levin M, Ailsworth SM, Commins SP, Platts-Mills TAE. Tick bites, IgE to
433 galactose- α -1,3-galactose and urticarial or anaphylactic reactions to mammalian meat:
434 The alpha-gal syndrome. *Allergy: European Journal of Allergy and Clinical Immunology*. John
435 Wiley and Sons Inc; 2024.
- 436 34. Barello C, Garoffo LP, Montorfano G, Zava S, Berra B, Conti A, et al. Analysis of major proteins
437 and fat fractions associated with mare's milk fat globules. *Mol Nutr Food Res*.
438 2008;52(12):1448–56.
- 439 35. Molloy MP, Phadke ND, Chen H, Tyldesley R, Garfin DE, Maddock JR, et al. Profiling the
440 alkaline membrane proteome of *Caulobacter crescentus* with two-dimensional
441 electrophoresis and mass spectrometry. *Proteomics*. 2002;2(7):899–910.
- 442 36. Wessel D, Flügge UI. A method for the quantitative recovery of protein in dilute solution in
443 the presence of detergents and lipids. *Anal Biochem* [Internet]. 1984 Apr 1 [cited 2020 Mar
444 17];138(1):141–3. Available from:
445 <https://www.sciencedirect.com/science/article/abs/pii/0003269784907826>
- 446 37. TRETTER V, ALTMANN F, MÄRZ L. Peptide-N₄-(N-acetyl- β -glucosaminyl)asparagine amidase
447 F cannot release glycans with fucose attached α 1 \rightarrow 3 to the asparagine-linked N-
448 acetylglucosamine residue. *Eur J Biochem*. 1991;199(3):647–52.

- 449 38. Cirrincione S, Aiuto B, Gosso E, Schiavone C, Portesi C, Rossi AM, et al. Proteomic study of
450 walnut oleosome and first evidence on oleosin sensitization in allergic patients. *Journal of*
451 *Food Composition and Analysis*. 2023;121(March):105386.
- 452 39. Candiano G, Bruschi M, Musante L, Santucci L, Ghiggeri GM, Carnemolla B, et al. Blue silver:
453 A very sensitive colloidal Coomassie G-250 staining for proteome analysis. *Electrophoresis*.
454 2004;25(9):1327–33.
- 455 40. Loprinzi Brauer CE, Motosue MS, Li JT, Hagan JB, Bellolio MF, Lee S, et al. Prospective
456 Validation of the NIAID/FAAN Criteria for Emergency Department Diagnosis of Anaphylaxis. *J*
457 *Allergy Clin Immunol Pract*. 2016;4(6):1220–6.
- 458 41. Binder AM, Cherry-Brown D, Biggerstaff BJ, Jones ES, Amelio CL, Beard CB, et al. Clinical and
459 laboratory features of patients diagnosed with alpha-gal syndrome—2010–2019. *Allergy:*
460 *European Journal of Allergy and Clinical Immunology*. 2023;78(2):477–87.
- 461 42. Sato T, Takio K, Kobata A, Greenwalt DE, Furukawa K. Site-Specific Glycosylation of Bovine
462 Butyrophilin. *The Journal of Biochemistry*. 1995;117(1):147–57.
- 463 43. Hvarregaard J, Andersen MH, Berglund L, Rasmussen JT, Petersen TE. Characterization of
464 glycoprotein PAS-6/7 from membranes of bovine milk fat globules. *Eur J Biochem*.
465 1996;240(3):628–36.
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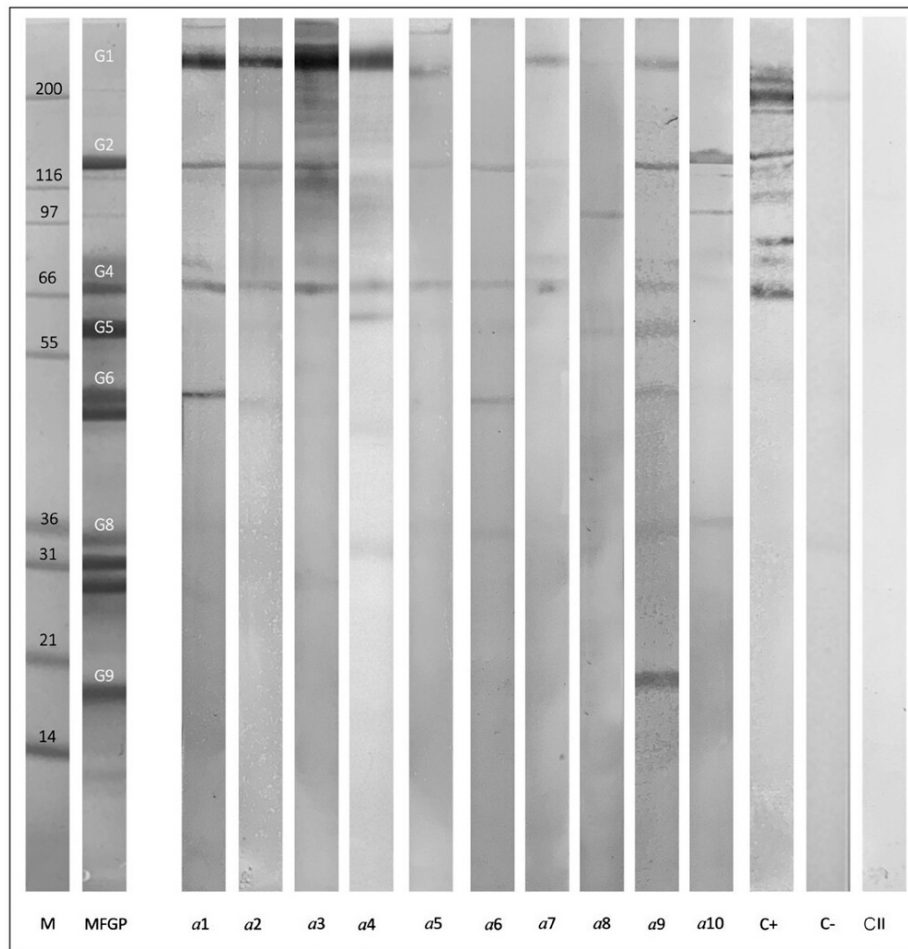
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472 **Figure 1.** Investigation of the three bovine milk fractions: caseins (CAS), whey proteins (WP) and
 473 milk fat globule associated proteins (MFGP). Panel A: LDS page of MFGP, WP and CAS. Panel B:
 474 immunoblotting of MFGP, WP and CAS with the anti- α -Gal IgG antibody. Panel C: immunoblotting
 475 of MFGP, WP and CAS with the sera of a pool of 10 AGS patients. M: molecular weight markers; C+:
 476 thyroglobulin; CII: secondary antibody control.



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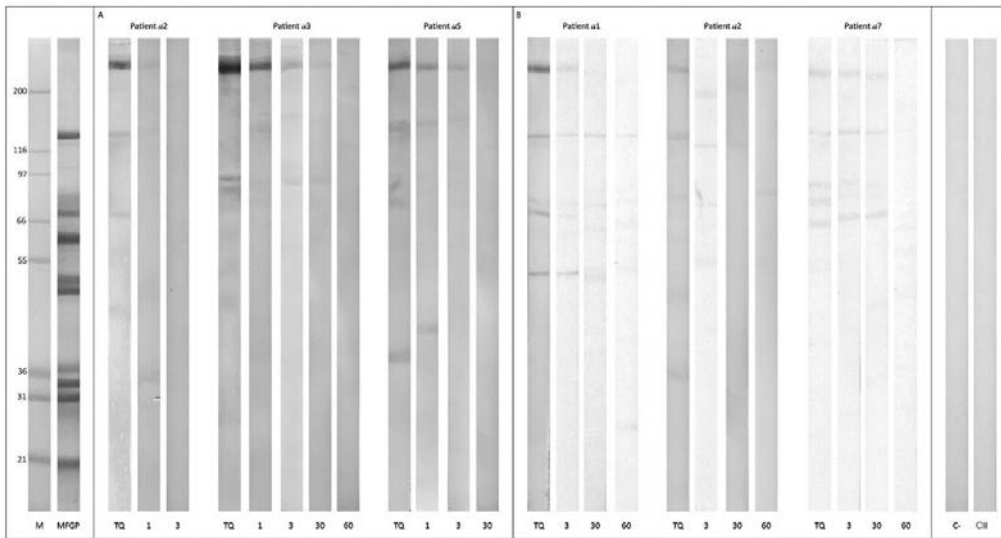
478 **Figure 2.** Investigation of α -gal bovine milk proteins. Panel A: LDS page of milk fat globule associated
 479 proteins (MFGP). MFGP enriched by means of incubation with beads bound with anti- α -gal IgG
 480 (MFGPb) and MFGPb de-glycosylated with PNGase for 3 hours (MFGPbDEG) and overnight (ON)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



484

485 **Figure 3.** Recognition of milk fat globule associated proteins (MFGP) by α -gal syndrome (AGS)
 486 patients. Immunoblotting of MFGP with the sera of 10 AGS patients (from α 1 to α 10). M: molecular
 487 weight marker; C+: thyroglobulin. C-: patient not assuming meat. negative control CII: secondary
 488 antibody control.

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490

491 **Figure 4.** Immunoprecipitation experiments of α -gal syndrome (AGS) patient's sera. Panel A:
 492 Immunoblotting of milk fat globule associated proteins (MFGP) with the sera of three patients (α 2.
 493 α 3. α 5) immunoprecipitated with different concentrations of thyroglobulin (1. 3. 30. and 60 μ g).
 494 Panel B: Immunoblotting of MFGP with the sera of three patients (α 1. α 2. α 7) immunoprecipitated
 495 with different concentrations of bovine xanthine oxidase (3, 30, and 60 μ g). M: molecular weight
 496 marker; C-: patient not assuming meat; CII: secondary antibody control.

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503 **Table 1.** Patients enrolled in the study.

PATIENT ID	SEX	AGE	CULPRIT FOOD	ALPHA GAL IgEs (KUA/L)
Alpha1	M	37	Since 2020: red meat	3.08
Alpha2	F	74	2017: veal kidney	0.37
			2018: tripe	
Alpha3	F	69	2017: boiled meat and soup with beef broth	86.50
			2018: offal	
			2019: veal broth.	
Alpha4	M	68	Since 2018: offal	15.01
			2022: stuffed meat	
Alpha5	F	74	2017: lamb stew	2.54
			2018: lamb liver and lung	
Alpha6	M	66	2018: "capocollo"	11.60
			3 more similar episodes after ingestion of pork or offal	
Alpha7	M	57	2010-2014: gummy bears	>100
			2016: red meat	
			2017: rabbit liver	
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

504

505 F: female; M: male.

506

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

N° BAN D	ENTRY	PROTEIN NAME	MW _{EXP} / MW _{THEOR} [DA]	PROTEIN SCORE>3 5	N° OF MATCHIN G PEPTIDES (>3)
G1	P80457	Xanthine oxidase	300000/1423 30	130,48	16
	Q8WNR8	Perilipin	300000/4525 1	52,718	8
	Q27960	Sodium-dependent phosphate transport protein 2B	300000/7582 5	52,365	5
	Q4GZT4	ATP-binding cassette transporter ABCG2	300000/7272 4	43,296	7
G2	P80457	Xanthine oxidase	130000/1467 90	317,14	30
	P18892	Butyrophilin subfamily 1 member A1	130000/5923 1	66,673	11
G3	G5E5T5	Ig-like domain-containing protein	80000/55968	129,03	10
	A0A3Q1M19 3	Glycoprotein 2 Butyrophilin subfamily 1 member	80000/58465	260,53	8
	P18892	A1	80000/59276	145,46	10
	C7FE01	Lactoferrin	80000/80278	55,906	8
G4	P81265	Polymeric immunoglobulin receptor	68000/82434	211,62	18
	A0A3Q1M19 3	Glycoprotein 2 Butyrophilin subfamily 1 member	68000/58465	92,215	10
	P18892	A1	68000/59276	106,11	15
	P26201	Glycoprotein IIIb	68000/46055	91,212	6
	G5E513	Ig-like domain-containing protein	68000/48107	95,157	9
	A0A3Q1LWT 4	Acyl-CoA synthetase long chain family member 1	68000/81442	79,564	10
	J7K1V4	Lactoferrin	68000/80278	75,774	12
	F1MHI1	Perilipin	68000/45281	53,926	7
	A0A3Q1MK3 8	Terpene cyclase/mutase family member	68000/74156	52,104	5
	G5	P18892	Butyrophilin subfamily 1 member A1	60000/59276	252,71
Q95114		Milk fat globule-EGF factor 8 protein (Lactadherin)	60000/43140	50,477	6

G6	Q95114	Milk fat globule-EGF factor 8 protein (Lactadherin)	51000/43140	198,57	22
	Q9TUM6	Perilipin-2	51000/49368	189,24	19
G7	Q95114	Milk fat globule-EGF factor 8 protein (Lactadherin)	49000/43140	231,35	13
	Q8HZM7	Perilipin	49000/45281	55,801	4
G8	P02663	Alpha-S2-casein	34000/26018	41,439	6
	P18892	Butyrophilin subfamily 1 member A1	34000/59231	47,768	5
G9	B5B0D4	Major allergen beta-lactoglobulin	19000/19969	116,59	11
	Q5E9I6	ADP-ribosylation factor 3	19000/20601	47,494	7
G10	P80457	Xanthine oxidase	300000/1423		
			30	37,778	5
G11	P80457	Xanthine oxidase	170000/1423		
			30	97,052	11
G12	P80457	Xanthine oxidase	130000/1467		
			90	167,66	20
G13	P80457	Xanthine oxidase	116000/1423		
			3	103,51	11
G14	G5E5T5	Immunoglobulin heavy constant mu	80000/56043	157,78	12
	F1MZQ4	Butyrophilin subfamily 1 member A1	80000/59231	65,44	7
G15	A0A4W2DW X4	Butyrophilin subfamily 1 member A1	60000/59245	94,962	13
G16	P0DOX5	Immunoglobulin gamma-1 heavy chain	53000/49328	97,277	10
G17	P01834	Immunoglobulin kappa constant	28000/11765	59,743	5
G18	P80457	Xanthine oxidase	300000/1466		
			90	17,852	3
G19	P80457	Xanthine oxidase	130000/1467		
			90	292,24	32
G20	G5E513	Immunoglobulin heavy constant mu	60000/56043	84,106	10
	P81265	Polymeric immunoglobulin receptor	60000/82434	65,441	9
	P18892	Butyrophilin subfamily 1 member A1	60000/59276	51,22	8
G21	F1MZQ4	Butyrophilin subfamily 1 member A1	57000/59231	63,366	7
G22	P18892	Butyrophilin subfamily 1 member A1	55000/59231	143,59	16
G23	Q9TUM6	Perilipin-2	48000/49368	83,058	8
	P18892	Butyrophilin subfamily 1 member A1	48000/59276	45,368	6
G24	Q95114	Milk fat globule-EGF factor 8 protein (Lactadherin)	44000/43140	137,39	16

G25	P21163.2	asparagine amidase PNGase F	40000/39032	227,36	16
	P18892	Butyrophilin subfamily 1 member A1	40000/59276	49,619	5
			130000/1467		
W1	P80457	Xanthine oxidase	90	323,31	20
	A0A4W2CZN		110000/1909		
W2	6	C3 complement	50	308,81	32
	A0A3Q1M3L		110000/4047		
	6	Ig-like domain-containing protein	5	106,25	7
W3	C7FE01	Lactoferrin	75000/76274	323,31	45
	G5E513	Ig-like domain-containing protein	75000/48107	307,5	16
	G3X6N3	Serotransferrin	75000/77738	117,08	22
	P80025	Lactoperoxidase	75000/71350	187,39	22
	A0A3Q1M3L				
	6	Ig-like domain-containing protein	75000/40475	44,51	4
	B3VTM3	Lactotransferrin	75000/78056	45,075	7
W4	P81265	Polymeric immunoglobulin receptor	68000/82434	134,53	11
	A0A4W2DZO		68000/77738		
	9	Serotransferrin		133,09	15
	E1BMJ0	Serpin family G member 1	68000/51772	95,139	5
	A0A4W2CZN		68000/19095		
	6	C3-beta-c	0	60,754	10
	A0A3Q1M03		68000/40475		
2	Ig-like domain-containing protein		79,946	4	
	A0A4W2DDL		68000/68198		
	5	Albumin		60,754	8
W5	P02769	Albumin	60000/68198	323,31	41
	A0A4W2CZN		60000/19095		
	6	C3 complement	0	244,45	28
	A0A3Q1M3L				
W6	6	Ig-like domain-containing protein	50000/40475	148,91	10
	G3NOV0	Ig-like domain-containing protein	50000/35951	49,249	6
	Q9TTE1	Serpin A3-1	50000/46236	75,075	7
W7	P00711	Alpha-lactalbumin	15000/14156	144,24	3
C1	P24627	Lactotransferrin	75000/78056	323,31	47
	P18892	Butyrophilin subfamily 1 member A1	75000/59276	41,743	3
C2	P02662	Alpha-S1-casein	27000/23689	323,31	8
	A0A140T8A9	Kappa-casein	27000/21237	190,77	4
	A0A452DHW				
	7	Beta-casein	27000/29221	62,074	5
	P02754	Beta-lactoglobulin	27000/19883	61,784	5

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

513

	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 (D₇₀₄NS)
	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)	N ₅₅ VS	yes (in cow)	not found	not found
	N ₂₁₅ VS	yes (in cow)	not found	215- 221 (D₂₁₅VS)
	N ₃₃₇ MT	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 (D₂₂₇NS)
	N ₃₉₀ NS	not	382-395 (N ₃₉₀ NS)	not found

514 N: asparagine; D: aspartic acid; MFGP: milk fat globule protein; XO: xanthine oxidase; BT:

515 butyrophilin; LA: lactadherin.

516