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1 Fetal glycosylation defect due to *ALG3* and *GOG5* variants detected via
2 amniocentesis: complex glycosylation defect with embryonic lethal phenotype

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21

22 AF, TK and EM designed the study. EM obtained funding. LMR and EM conducted the
23 consenting process. AF, RTS, EK, LMR, MJW, and EM reviewed the case. RTS, DU and TK
24 designed the experiments. RTS and WR conducted all experiments. AF and EK performed the
25 bioinformatic analysis. AF and RTS wrote the original draft. WR, DU, TK, EK, LMR, MJW and
26 EM reviewed the draft and made important intellectual contributions. EM obtained funding and
27 coordinated the study. EM made the final decision to submit the manuscript and is the guarantor
28 to this work.

29

30 Keywords: congenital disorders of glycosylation; osteochondrodysplasia; fetal cell research; fetal
31 demise; whole-exome sequencing

32 ABSTRACT

33 **Introduction:** Congenital disorders of glycosylation (CDG) are inborn errors of glycan
34 metabolism with high clinical variability. Only a few antenatal cases have been described with
35 CDG. Due to a lack of reliable biomarker, prenatal CDG diagnostics relies primarily on
36 molecular studies. In the presence of variants of uncertain significance prenatal glycosylation
37 studies are very challenging.

38 **Case Report:** A consanguineous couple had a history of second-trimester fetal demise with
39 tetralogy of Fallot and skeletal dysplasia. In the consecutive pregnancy, the second trimester
40 ultrasonography showed skeletal dysplasia, vermian hypoplasia, congenital heart defects,
41 omphalocele and dysmorphic features. Prenatal chromosomal microarray revealed a large region
42 of loss of heterozygosity. Demise occurred at 30 weeks. Fetal whole exome sequencing showed a
43 novel homozygous likely pathogenic variant in *ALG3* and a variant of uncertain significance in
44 *COG5*.

45 **Methods:** Western blot was used to quantify *ALG3*, *COG5*, *COG6*, and the glycosylation
46 markers ICAM-1 and LAMP2. RT-qPCR was used for *ALG3* and *COG5* expression in cultured
47 amniocytes and compared to age matched controls.

48 **Results:** *ALG3* and *COG5* mRNA levels were normal. ICAM-1, LAMP2, *ALG3* and *COG5*
49 levels were decreased in cultured amniocytes, suggesting the possible involvement of both genes
50 in the complex phenotype.

51 **Conclusion:** This is the first case of successful use of glycosylated biomarkers in amniocytes,
52 providing further options of functional antenatal testing in CDG.

53 INTRODUCTION

54 Congenital disorders of glycosylation (CDG) are a growing group of rare inherited metabolic
55 disorders that affect the building and processing of glycans. The clinical manifestations of CDG
56 vary depending on the biochemical defect (the impacted glycosylation step) and the molecular
57 background. Due to the extreme rarity of these disorders, for many CDG the full clinical
58 spectrum is not yet known; many of those disorders have a complex, multisystem phenotype
59 including malformations, early-onset metabolic abnormalities, and organ dysfunction. CDG are
60 classified primarily according to the type of glycosylation affected – N-glycosylation
61 (attachment of glycans to the amide group of asparagine residues in proteins) or O-glycosylation
62 (attachment of glycans to the lateral oxygen atom of serine or threonine residues). CDG affecting
63 N-glycosylation are further divided according to whether they affect the assembly of glycans in
64 the endoplasmic reticulum (CDG-I) or the remodeling of those glycans either in the endoplasmic
65 reticulum or the Golgi apparatus (CDG-II). With the implementation of next-generation
66 sequencing (NGS) in clinical practice, the knowledge of the genetic etiology of CDG has
67 expanded enormously with more than 137 different CDG discovered to date (1).

68 We report a consanguineous couple with two spontaneous pregnancy losses in which the
69 fetuses presented with a similar abnormal phenotype. Whole exome sequencing (WES) of
70 amniocyte DNA obtained from the second miscarriage identified a homozygous missense variant
71 in *COG5* and a homozygous nonsense variant in *ALG3* that were also confirmed by PCR in the
72 first miscarriage. These two genes are included in the N-glycosylation pathway and potentially
73 contributory to fetal demise (2-4). However, both variants were novel and therefore further
74 research testing was pursued.

75

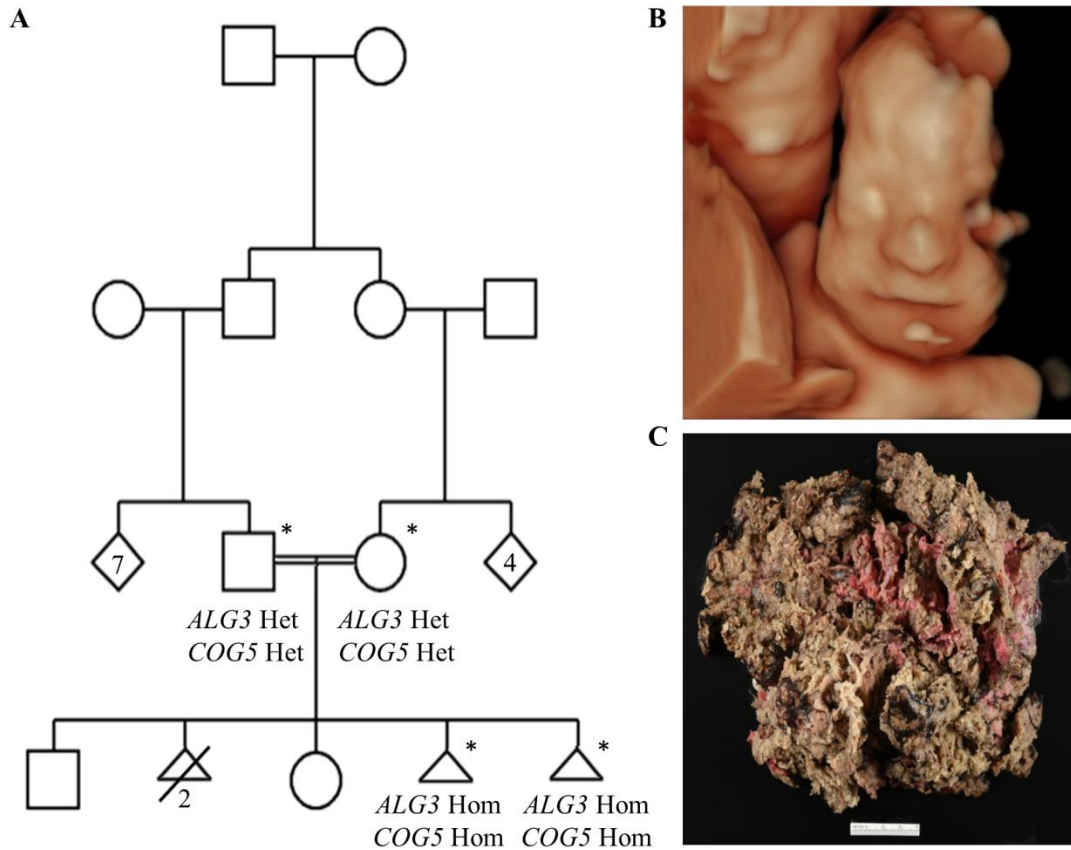
76 CASE REPORT

77 A 28-year-old G6P2122 woman at 12 3/7 weeks gestational age presented for genetic
78 counseling due to a history of previous fetal demise, family history of spinal muscular atrophy,
79 and consanguinity (figure 1A). She and her partner, both of Pakistani ancestry, are the parents of
80 two healthy children, aged 8 and 5 years. The couple also had a pregnancy which resulted in
81 intrauterine fetal demise at 24 4/7 weeks; autopsy revealed tetralogy of Fallot, probable skeletal
82 dysplasia with decreased long bone length (<2nd percentile for gestational age), and dysmorphic
83 findings including micrognathia, slit-like nostrils, and flat upper lip. Chromosomal microarray
84 (CMA) was negative for deletions or duplications, but revealed large regions with loss of
85 heterozygosity (LOH) presumably due to consanguinity (approximately 8%). The patient's
86 obstetrical history also included two first trimester elective terminations.

87 At 17 weeks gestation of the current pregnancy, early anatomical ultrasound revealed similar
88 anomalies to the prior affected pregnancy (figure 1B) including skeletal dysplasia with
89 shortening of all long bones, short ribs, micrognathia, small nose, vermian hypoplasia, and
90 congenital heart defect. The fetus had the additional finding of an omphalocele and echogenic
91 bowel. Amniocentesis was performed and CMA was negative for deletions and duplications, but
92 revealed 15% LOH.

93 At 30-1/7 weeks gestational age, the patient experienced preterm onset of labor resulting in
94 delivery of a stillborn infant. The placenta was large-for-gestational-age, friable, and hydropic on
95 pathological examination (figure 1C). WES performed on amniocytes from this second
96 pregnancy identified two homozygous variants of interest (*COG5* c.944C>G, p.Ser315Cys; and
97 *ALG3* c.1188G>A, p.Trp396*) inherited from both healthy, heterozygous carriers parents.

98 Consequently, placental tissue from the first pregnancy was tested by directed PCR and found to
99 also carry both variants.



100

101 Figure 1: A) Pedigree of the family showing a high degree of consanguinity. Asterisks indicate
102 individuals genetically tested. The genotypes for the variants in *ALG3* and *COG5* are indicated
103 under each symbol. Het: heterozygous, Hom: homozygous. B) Tridimensional reconstruction of
104 fetal ultrasound at gestational age 24 weeks, 6 days showing hypertelorism, depressed nasal
105 bridge, and thin upper lip of the fetus. C) Photograph of the fetal surface of the placenta, showing
106 hydropic, partially disintegrated villi.

107

108 METHODS

109 **Whole Exome Sequencing Analysis**

110 WES from the second miscarriage and both parents was performed at the Clinical Genomics
111 Laboratory (Mayo Clinic). Paired-end libraries were prepared using 1.0 µg of genomic DNA
112 using the Agilent Bravo liquid handler (Agilent) as indicated by the manufacturer. Whole exon
113 capture was carried out using 750 ng of the prepped library following the protocol for Agilent's
114 SureSelect Human All Exon v5 + UTRs 75 MB kit. The purified capture products were
115 amplified using the SureSelect Post-Capture Indexing forward and Index PCR reverse primers
116 (Agilent) for 12 cycles. Libraries were sequenced at an average coverage of ~80X following
117 Illumina's standard protocol in an Illumina cBot and HiSeq 3000/4000 PE Cluster Kit. The flow
118 cells were sequenced as 150 X 2 paired end reads on an Illumina HiSeq 4000 using HiSeq
119 3000/4000 sequencing kit and HCS v3.3.52 collection software. Base-calling is performed using
120 Illumina's RTA version 2.7.3. Data was processed through an in-house bioinformatics pipeline
121 and analyzed using Ingenuity (Qiagen) by the Center for Individualized Medicine (Mayo Clinic).

122 **Variant Segregation Testing**

123 A formalin-fixed, paraffin embedded (FFTE) sample from the placenta of the first
124 miscarriage was used for testing and was used to obtain genomic DNA following a standard
125 clinical procedure. The presence of both the homozygous *COG5* and *ALG3* variants found in the
126 second miscarriage was tested by PCR using the primers described in Table 1 and the Platinum
127 TaqDNA polymerase High Fidelity commercial kit (ThermoFisher Scientist) following the
128 manufacturer indications. The conditions for the melting step were 51°C (for *COG5*) or 53°C (for
129 *ALG3*) for 30 s.

130 **Table 1.** Primers used for targeted sequencing of *COG5* and *ALG3* variants and mRNA
 131 expression levels by RT-PCR .

Gene Symbol	Test	Oligonucleotide Sequence	Orientation	Predicted Size
<i>COG5</i>	Family segregation of c.944C>G variant	5'-CTCAATAAATTATTTCTAAAGAAGGA-3'	Forward	465 bp
		5'-CAATACTTTTTGTAGATGTTGTACCT-3'	Reverse	
<i>ALG3</i>	Family segregation of c.1188G>A variant	5'-CATACAGATCGTTTCTACCCTCT-3'	Forward	446 bp
		5'-GTGGGCTTCTTGCTGT-3'	Reverse	
<i>COG5</i>	mRNA expression	5'- TGGGTCCATTCTGTAGACGA-3'	Forward	N/A
		5'- GTTCACTTGCCTGGAAGAGC-3'	Reverse	
<i>ALG3</i>	mRNA expression	5'-CACCTTCTGGGTCATTACAGG-3'	Forward	N/A
		5'- GTGTCACCCTGCAGTTGGGTATAGT-3'	Reverse	
<i>RNA18S</i>	mRNA expression	5'-GTAACCCGTTGAACCCCAT-3'	Forward	N/A
		5'-CCATCCAATCGGTAGTAGCG-3'	Reverse	
<i>GAPDH</i>	mRNA expression	5'-GCCAAAAGGGTCATCATCTC-3'	Forward	N/A
		5'-GGCCATCCACAGTCTTCT-3'	Reverse	
<i>ACTB</i>	mRNA expression	5'-CATGTACGTTGCTATCCAGGC-3'	Forward	N/A
		5'-CTCCTTAATGTCACGCACGAT-3'	Reverse	

132

133 Amniocyte Culture

134 Amniocytes were obtained from the amniocentesis during the second pregnancy. Primary
 135 cultures were established for 9 days before transferring to T25 culture flasks. Cells were
 136 incubated at 37°C with 5% CO₂, 5% O₂ and 90% N₂ in 50% Chang C Working Medium (Irvine)
 137 and 50% Dulbecco's Modified Eagle Medium Alpha with GlutaMAX (Gibco) supplemented
 138 with 12.5% fetal bovine serum (Gibco) and 1% Penicillin/Streptomycin solution (Gibco).

139 Western blot

140 Protein extracts from amniocytes were denatured for either 30 minutes at 70°C (ICAM-1 and
 141 LAMP2) or 5 minutes at 95°C (ALG3, COG5 and COG6) in dithiothreitol (DTT) or β-
 142 mercaptoethanol and LDS-containing NuPAGE sample buffer (Novex and Invitrogen, Carlsbad,
 143 CA, USA) and loaded into either a 10% (ICAM-1 and ALG3) or a 4-12% (LAMP2, COG5, and
 144 COG6) Bis-Tris gel (Invitrogen). Electrophoresis was performed in MOPS SDS NuPAGE

145 running buffer (Novex) at 200V for 60 minutes, followed by transfer to a nitrocellulose
146 membrane (Bio-Rad laboratories, Germany) in NuPAGE transfer buffer (Novex) at 35V for 180
147 minutes. Membranes were blocked at either SEA Block blocking buffer (Thermo Scientific,
148 Rockford, IL, USA) (ICAM-1) or 5% blocking-grade non-fat milk (Sigma Aldrich, Saint Louis,
149 MO, USA) or 5% BSA in Tris buffered saline with 0.1% Tween-20 (TBST). Primary antibodies
150 (ICAM-1 mouse monoclonal, Santa Cruz Biotechnology, 1:500; LAMP2 mouse monoclonal,
151 H4B4 clone, Invitrogen, 1:1000; ACTB rabbit monoclonal antibody, ABclonal, 1:10,000;
152 GAPDH mouse monoclonal antibody, Invitrogen, 1:20,000; ALG3 rabbit polyclonal, 1:125,
153 Abnova; COG5 rabbit polyclonal and COG6 rabbit polyclonal provided in house (5), University
154 of York, UK, 1:500) were incubated overnight or 48-72 hours at 4°C and washed with 0.1%
155 Tween-20 in PBS or TBST (Fisher Bioreagents, Fair Lawn, NJ, USA). For ICAM-1, LAMP2,
156 ALG3, ACTB and GAPDH, a fluorescent-conjugated secondary antibody (donkey anti-mouse
157 cross-adsorbed secondary antibody, DyLight 800 conjugate; donkey anti-rabbit cross-adsorbed
158 secondary antibody, DyLight 680 conjugate; both from Invitrogen) was used. For COG5 and
159 COG6, a biotinylated secondary antibody (Biotin-SP-conjugated AffiniPure donkey anti-rabbit,
160 Jackson ImmunoResearch Laboratories, West Grove, PA, USA, 1:1000) was used. Secondary
161 antibodies were incubated for 1 hour at 4°C, washed with 0.1% Tween-20 in PBS, and
162 membranes were either detected and quantified in an Odyssey Fc system (Li-Cor Biosciences,
163 Lincoln, NE, USA) or incubated in fluorophore-conjugated streptavidin (AlexaFluor 680-
164 conjugated streptavidin, Jackson ImmunoResearch Laboratories, 1:1000) for 30 min at 4°C.
165 After incubation with streptavidin, membranes were washed with 0.1% Tween-20 in PBS,
166 detected and quantified in the same Odyssey Fc system.

167 **Real-time quantitative polymerase chain reaction (RT-qPCR)**

168 Isolated RNA from amniocytes was reversed transcribed to cDNA using the SuperScript III
169 First-Strand kit (Invitrogen) according to the manufacturer's protocol (primer sequences were
170 synthesized by Integrated DNA Technologies, Coralville, IA, USA and are available in Table 1).
171 The reverse-transcriptase polymerase chain reaction (RT-PCR) was set up using the following
172 conditions: 65°C for 5 minutes, 25°C for 10 minutes, 50°C for 50 minutes, 85°C for 5 minutes.
173 For the real-time quantitative polymerase chain reaction (RT-qPCR) mixtures contained each
174 6.05 µL SYBR Green (Applied Biosystems, Warrington, UK), 3.63 µL double deionized H₂O,
175 1.21 µL of the respective primers, and 1.1 µL of cDNA. RT-qPCR cycles were performed and
176 read in a LightCycler 480 II (Roche Molecular Systems, Rotkreuz, Switzerland) as follows: pre-
177 incubation at 95°C for 5 minutes; 45 cycles of amplification at 95°C for 10 seconds, 60°C for 10
178 seconds, 72°C for 10 seconds; melting curve at 95°C for 5 seconds and 65°C for 1 minute; and
179 cooling at 40°C for 30 seconds.

180 RESULTS

181 Genetic Analysis

182 The analysis of WES data focused on the overlapping LOH areas observed by CMA in both
183 miscarriages, where only three genes (*EDEM3*, *TMEM140* and *COG5*) were found to carry
184 homozygous rare (below 1% in healthy population (6)) variants. An in-depth review of these
185 results concluded that the variant in *COG5* (c.944C>G; p.Ser315Cys) potentially explained the
186 patient's phenotype. Interestingly, further review identified an additional gene in *N*-glycosylation
187 pathway (*ALG3* – c.1188G>A; p.Trp396*) carrying a homozygous likely pathogenic variant.

188 The homozygous c.944C>G; p.Ser315Cys variant in *COG5* is a novel variant not reported in
189 the healthy population (gnomAD (6)) or variants databases (ClinVar and HGMD). The amino

190 acid position is not conserved across species and it is not located in any functional domain
191 described. Some *in silico* predictions suggested a deleterious effect (SIFT, PolyPhen,
192 MutationTaster) although in other cases (M-CAP) was predicted as benign. The constraint values
193 described in gnomAD for this gene indicate tolerance to missense variation. We classified the
194 variant as uncertain significance (VUS) according to ACMG criteria but it was considered
195 relevant due to the possible phenotypic overlap between the two fetuses and other CDG patients
196 including skeletal dysplasia.

197 On the other hand, the homozygous c.1188G>A; p.Trp396* variant in *ALG3* is classified as
198 likely pathogenic by ACMG criteria but the possible connection with the phenotype observed
199 was not clear. This variant was not present in the healthy population (gnomAD (6)) or variant
200 databases (ClinVar and HGMD). The variant is located in the last exon of the protein and does
201 not impact any functional domain described. It is not predicted to initiate nonsense mediated
202 decay by the 50bp rule but the constraint values described in gnomAD for this gene indicate
203 intolerance to loss of function variations.

204 Both variants were also detected in the first miscarriage after testing the genomic DNA
205 obtained from a formalin-fixed, paraffin embedded (FFTE) sample by PCR. Unfortunately,
206 healthy siblings were not available for testing.

207 **Glycosylation markers**

208 N-glycosylation markers ICAM1 and LAMP2 analyzed by Western blot were both abnormal
209 in cultured amniocytes from the affected individual compared to cultured amniocyte controls,
210 confirming a glycosylation defect. Decreased abundance of glycosylated ICAM-1 was detected
211 in cultured amniocytes (the protein expression was 61.4% lower than that in control amniocytes),

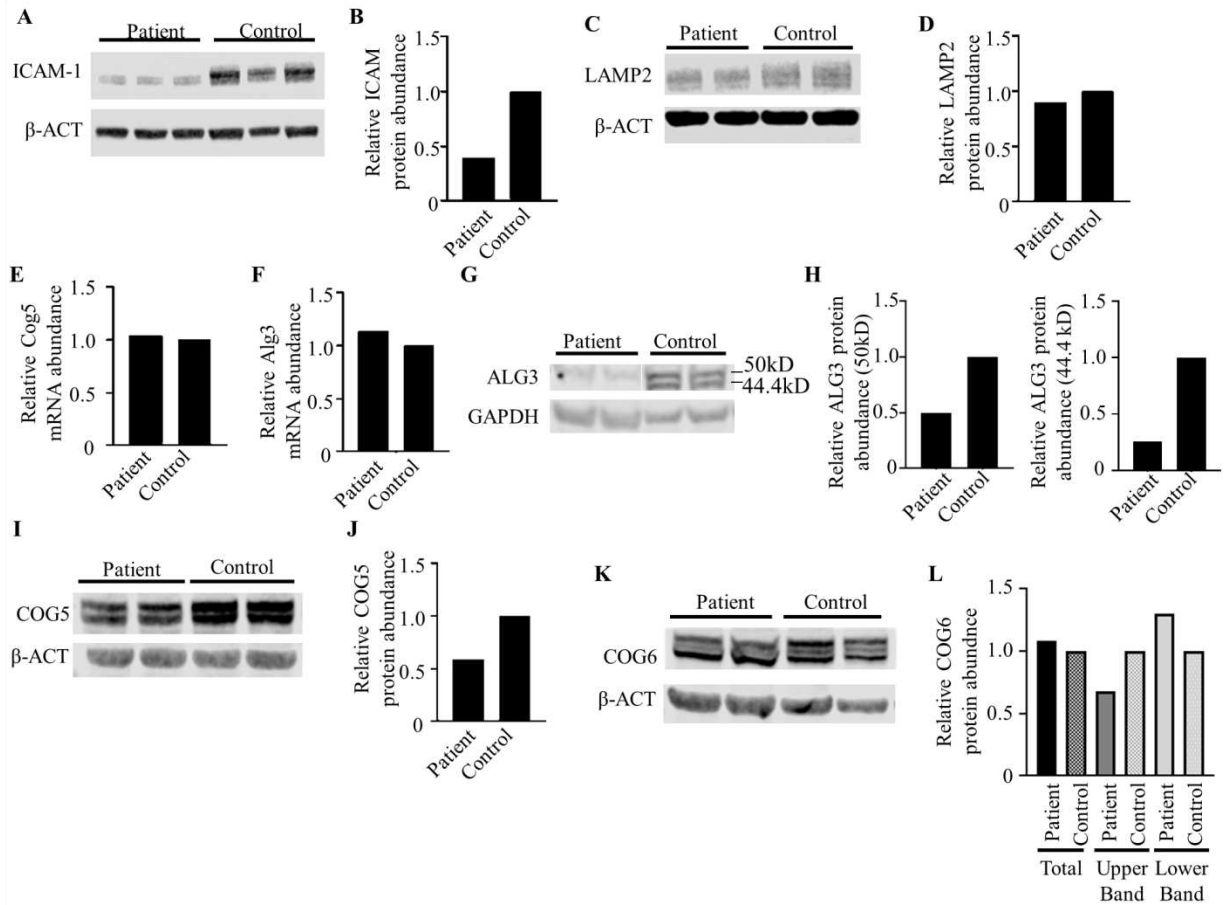
212 as shown in figure 2A and B. The LAMP2 protein showed an abnormal migration pattern by
213 Western blot, compatible with abnormal LAMP2 glycosylation in the affected individuals
214 amniocytes compared to controls (figure 2C and D).

215 **Gene expression of *ALG3* and *COG5***

216 Gene expression studies by RT-qPCR showed no decrease in the level of gene products for
217 either *ALG3* or *COG5* genes, additionally excluding nonsense-mediated decay in the case of
218 *ALG3*. Levels of *Cog5* mRNA were similar between affected individual and control amniocytes
219 (figure 2E) while *Alg3* mRNA levels were slightly higher in the affected individual's amniocytes
220 (figure 2F).

221 **Protein expression of *ALG3*, *COG5* and *COG6***

222 Protein expression of *ALG3*, *COG5* and *COG6* (another subunit from the same COG lobe)
223 were all decreased in the affected individual's amniocytes compared to the control amniocytes.
224 The *ALG3* Western blot showed two bands corresponding to the two different isoforms of the
225 protein (50kD and 44kD) that were both decreased by 49.8% and 74.3% respectively (Figure 2 G
226 and H). Quantitation of *COG5* protein was found 41% lower compared to the control by Western
227 blot, as shown in figure 2J. Similarly, Western blot analysis of *COG6* showed a double band
228 (figure 2K and L): the upper band, which is appropriate for the predicted size of 68 kDa for
229 *COG6*, was 34% decreased in the affected individual's amniocytes compared to controls. The
230 lower band was 30% increased in the affected individual's amniocytes compared to controls
231 (figure 2L). We hypothesize that this band may be the product of proteolytic degradation of
232 *COG6*.



233

234 Figure 2: A) Representative Western blot of patient and control amniocytes against ICAM-1. B)

235 Quantification of Western blot shown in A indicating protein abundance 61.4% lower in the

236 patient, consistent with a glycosylation defect. C) Representative Western blot of patient and

237 control amniocytes against LAMP2. D) Quantification of Western blot shown in C indicating

238 protein abundance 11.4% lower in the patient, as well as an altered protein migration pattern in

239 the patient compared to control, consistent with a glycosylation defect. E) RT-qPCR of Cog5 in

240 patient amniocytes and control amniocytes, averaged against the three housekeeping transcripts

241 Rna18s, Gapdh, and Bact, showing approximately same levels of Cog5 mRNA. F) RT-qPCR of

242 Alg3 in patient amniocytes and control amniocytes, averaged against the three housekeeping

243 transcripts Rna18s, Gapdh, and Bact, showing an increase of 13.3% in mRNA abundance in the

244 patient. G) Representative Western blot of ALG3 expression in patient amniocytes and control
245 amniocytes. Two bands are present (50kD and 44.4 kD) corresponding to each isoform of ALG3.
246 H) Quantification of Western blot shown in G indicating a protein abundance 49.8% (50kD
247 band) and 74.3% (44.4 kD) lower in the patient compared to control amniocytes. I)
248 Representative Western blot against COG5 in patient amniocytes and control amniocytes. J)
249 Quantification of Western blot shown in I indicating a protein abundance 41% lower in the
250 patient compared to control amniocytes. K) Representative Western blot performed in patient
251 and control amniocytes against COG6. L) Quantification of I showing a lower intensity of the
252 upper band and a higher intensity of the lower band compared to control amniocytes. β -ACT: β -
253 actin; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase.

254

255 DISCUSSION

256 CDG are rare metabolic disorders that affect the assembly and processing of glycans, mainly
257 in the endoplasmic reticulum (ER) and in the Golgi apparatus (7). The CDG group is extremely
258 heterogeneous, ranging from diseases that are tissue-restricted (8) to multi-system organ
259 involvement (9). In most rare CDG our current knowledge of the phenotypic spectrum is biased
260 because it often relies on reports of a single or a few cases. CDG with early fetal loss thus tend to
261 be underdiagnosed and underreported. For example, COG5-CDG, first described as a mild
262 psychomotor delay syndrome (10), was reported in subsequent reports in patients with prenatal
263 features who develop significant complications in multiple systems (11). This high variability
264 also complicates the identification of the genetic cause resulting in the different manifestations of
265 the disease.

266 Here we report on a consanguineous family with two spontaneous fetal demises showing
267 similar timeframes and phenotype including a heart defect and signs of skeletal dysplasia. WES
268 testing revealed two variants in genes included in the glycosylation pathway (homozygous VUS
269 in *COG5* and homozygous likely pathogenic variants in *ALG3*) inherited from both parents who
270 were both heterozygous carriers. Since both variants were confirmed by PCR to be also present
271 in homozygosis in the first miscarriage, we hypothesized that the phenotype was caused by a
272 glycosylation defect. We evaluated the N-glycosylation pathway in cultured amniocytes obtained
273 from the second pregnancy by measuring two common glycosylation markers using Western
274 blot: ICAM-1 and LAMP2. The expression of both proteins was abnormal compared to control
275 amniocytes (Figure 2A-C). This combined deficit is expected in most CDG, even though
276 decreased abundance of LAMP2 or ICAM-1 in isolation do not always reflect major expression
277 changes in all CDG (12, 13). We performed RT-qPCR studies of gene expression of *COG5* and
278 *ALG3* that indicated normal mRNA levels (Figure 2E and F), suggesting that the presence of the
279 variants did not affect gene expression and a possible alteration would be at the protein level.

280 The protein levels of *ALG3* and *COG5* in the affected individual's sample by Western blot
281 were markedly reduced compared to controls (Figure 2G to J). The reduction of *ALG3* protein
282 levels could be explained by a higher degradation of the truncated protein as a consequence of
283 the variant, which is supported by the *ALG3* mRNA levels being compared to the controls. In the
284 case of *COG5* we postulated that the missense variant could interfere with the formation of lobe
285 B of the COG complex, causing the unassembled protein to be degraded impairing the
286 glycosylation pathway in these individuals. This idea was tested by measuring the presence of
287 *COG6* (another COG protein) in the same samples. *COG6* was present in two distinct molecular
288 weights (figure 2K and L), with a decrease of the heavier form and an increase in the lighter

289 form. It is possible that this happens due to an increased degradation of COG6 and, being COG6
290 a COG subunit located in the same lobe but not in direct contact with COG5, this suggests the
291 destabilization of the whole lobe B by a mutated COG5. This is in accordance with experiments
292 by Rymen and colleagues showing decreased expression of COG7 (another lobe B subunit) in
293 COG5-CDG (11).

294 With this data we concluded that the probable cause for both fetal losses was a congenital
295 defect of glycosylation. We show evidence that the *ALG3* truncating variant results in decreased
296 protein level, and the *COG5* variant is probably impairing the formation of the COG complex.
297 Therefore, although both defects individually could lead to a CDG phenotype, we cannot rule out
298 an effect in the pathway following a “multiple hit” mechanism of disease impacting the N-
299 glycosylation pathway, starting with the first insult in the ER-related assembly. Although the
300 presence of the protein is not a guarantee for protein function, it is possible that some residual
301 *ALG3* activity remains and therefore some glycosylated proteins could be transferred to the
302 Golgi for further processing. There, the additional COG defect would interfere with Golgi
303 trafficking, adding a second biochemical abnormality to already decreased glycosylation. We
304 hypothesize that this could cause truncated glycans similar to a "digenic" mechanism.

305 If this hypothesis is true, we would expect a combined type I/type II glycosylation defect in
306 our patient, similar to that seen in PGM1-CDG. It is interesting to note that our patient had a
307 prenatal presentation of skeletal long bone shortening/short stature, congenital heart
308 malformation and micrognathia, which are also features of PGM1-CDG (14, 15) and have been
309 observed in some of the COG deficiencies, like COG7-CDG (16).

310 In summary, we have provided evidence indicating a glycosylation defect in this family that
311 may have impacted fetal development, leading to fetal demise. Interestingly, it is described that

312 protein glycosylation plays a major role in development and maintenance in the third trimester of
313 gestation – the period when both miscarriages occurred – also supporting this idea (2-4).
314 Additionally, we show for the first time that studies of glycosylated proteins such as ICAM-1
315 and LAMP2 can be carried out in cultured amniocytes in the third trimester, leading to the
316 development of more elaborate and elaborate glycoproteomics techniques in pregnancy losses
317 suspected with a diagnosis of CDG (17).

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330

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