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Heterogeneity of disease-causing variants in the Swedish galactosemia population: Identification of 16 novel *GALT* variants

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Abstract

The aim was to determine disease-causing variants in the *GALT* gene which codes for the enzyme galactose-1-phosphate uridylyltransferase. Loss of activity of this enzyme causes classical galactosemia—a life threatening, treatable disorder, included in the Swedish newborn screening program since 1967. A total of 66 patients with the disease are known in Sweden and 56 index patients were investigated. An additional two patients with Duarte galactosemia were included. The disease-causing variants were identified in all patients. As reported from other countries only a few variants frequently recur in severe disease. The two variants p.(Gln188Arg) (c.563A>G) and p.(Met142Lys) (c.425T>A) are present in several index patients whereas the remaining are found in one to three patients each. The most common variant, p.(Gln188Arg), has an allele frequency of 51% in the cohort. A total of 16 novel variants were found among the 33 different variants in the cohort. Two of these are synonymous variants affecting splicing, demonstrating the importance of the evaluation of synonymous variants at the cDNA level. Concise sentence: Galactosemia is a rare disease in Sweden and the disease-causing variants are heterogenous including two synonymous variants.

KEY WORDS

galactosemia, genetics, mutations, neonatal screening, newborn screening

1 | INTRODUCTION

Galactosemia is a group of autosomal recessive disorders: galactokinase deficiency (OMIM #230200), galactose-1-phosphate uridylyltransferase deficiency (OMIM #230400, *GALT* deficiency), galactose epimerase deficiency (OMIM #230350) and the recently discovered galactose mutarotase deficiency (*GALM*, OMIM #137030)¹ affecting the metabolism of galactose (Gal). The most common form is *GALT* deficiency resulting in an accumulation of Gal, galactose-1-phosphate (Gal-1-P), galactitol and galactonate.²

The disease is potentially lethal if not treated early with a Gal-restricted diet. Early diagnosis by newborn screening

(NBS) has improved the prognosis for patients. Long-term complications ranging from mild speech and language delay to more severe mental difficulties and neurological defects are not uncommon, and a high proportion of affected women have primary ovarian insufficiency.^{3,4}

Patients with a milder form of galactosemia are most often compound heterozygous for one severe, classical variant (G), and the milder, Duarte variant (D), p.(Asn314Asp) (c.940A>G). The p.(Asn314Asp) variant results in a residual activity of 50% when it is found in *cis* with the four-base deletion, c.-119_-116delGTCA in the promoter region, and the three intron variants c.378-27G>C, c.507+62G>A and c.508-24G>A.⁵ This combination of *cis* Duarte variants is well known and is found in

most studied populations. Patients with D/G are often detected in NBS programs due to a GALT activity of approximately 25% of normal.⁶ Although there is an uncertainty whether these patients need treatment, there are several publications indicating a favorable outcome for D/G patients without treatment with a Gal-restricted diet.⁷⁻⁹

The first screening method described was a bacterial inhibition assay,¹⁰ followed by the Beutler enzyme spot test.¹¹ The current methods employed by screening laboratories either measure the activity of the GALT enzyme (EC 2.7.7.12) and/or the amount of total Gal (Gal and Gal-1-P).¹² Galactosemia screening, including all three enzyme deficiencies, was added to the Swedish NBS program in 1967.¹³ The Beutler test was implemented in Sweden in 1986, and since then, the program only detects patients with GALT deficiency. The recall levels were adjusted in 1992 to minimize the detection of infants with the D/G variant.¹⁴

The Swedish NBS program is centralized to one laboratory, in Stockholm, with approximately 120 000 babies screened annually. Centralization of the screening facilitates record retention of all recalled cases, true as well as false positive. The fact that all Swedish citizens have a unique personal identification number, minimizes the risk that patients are lost to follow up.

The screening laboratory is part of the Centre for Inherited Metabolic Diseases at Karolinska University Hospital, Stockholm, one of two Swedish laboratories performing biochemical and molecular diagnostics of inborn errors of metabolism. This includes molecular genetic investigations, determination of GALT activity in erythrocytes, analysis of Gal and Gal-1-P in dried blood spots (DBS) and galactitol in urine.

The first sequence variants, causing classical galactosemia, were reported in 1991.^{15,16} The most cited variants in Caucasians are p.(Gln188Arg) (c.563A>G) and p.(Lys285Asn) (c.855G>T), while p.(Ser135Leu) (c.404C>T) is common among patients of African origin. These three variants account for more than 80% of all affected alleles reported.¹⁷ Patients harboring the variant p.(Ser135Leu) have absent GALT activity in erythrocytes, but approximately 10% residual activity in tissues such as liver, intestine and leukocytes, resulting in a milder clinical picture.¹⁸

Here we present the spectrum of *GALT* variants in the Swedish galactosemia population, including 16 variants not reported to HGMD Professional 2018.3 (HGMD) or the ARUP *GALT* database, (http://www.arup.utah.edu/database/galt/GALT_display.php, accessed 22 October 2018).¹⁹

2 | MATERIAL AND METHODS

2.1 | Patients

As at 31 December 2018, 5 331 800 newborns have been screened for galactosemia. Of these, 52 patients (29 males),

have been diagnosed with severe GALT deficiency (GALT activity <1% of normal) and five (two males) with mild GALT deficiency (GALT activity between 3% and 25% of normal and normal excretion of galactitol in urine from age 1 year). An additional nine patients (seven males) with classical galactosemia are known in the country: three patients diagnosed before the onset of screening and six in their home countries.

All galactosemia patients in Sweden, except one were screened for *GALT* variants in this or a previous study.²⁰ The genetic study enrolled index cases from 56 families (32 males): age 1-54, median 25.8 years. Of these families, eight have more than one child with galactosemia. An additional two patients with Duarte galactosemia, are included.

The patients detected by NBS, were diagnosed at an average age of 8 days (range 3-14 days) before 2008, and at an average age of 4 days (range 3-6 days) from 2008, onwards. In 2008 the sampling time was moved from as soon as possible after 72 hours to as soon as possible after 48 hours of age.¹⁴

Genotyping is part of the clinical diagnostic routine for patients with galactosemia. Variants were confirmed in both parents of 60% (34/56) of the patients.

The project was approved by the Regional Ethical Committee of Stockholm (2008/351-31).

3 | ANALYSIS OF THE GALT GENE

3.1 | DNA-sequencing

Genomic DNA was isolated from whole blood with QIAamp DNA blood mini/midi kit (QIAGEN, Venlo, The Netherlands). Search for variants was performed by Sanger sequencing of the promoter, exons and intron-exon flanking regions in eight fragments. Out of the 11 exons, 3-4, 5-6 and 8-9 were sequenced together. Direct-cycle sequencing of all PCR fragments was performed with BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA) following the manufacturer's recommendations. Nomenclature for variants follows the guidelines and recommendations of the Human Genome Variation Society (<http://www.HGVS.org/varnomen>)²¹ and novel variants were validated using the Mutalyzer program (<https://mutalyzer.nl/>). cDNA numbering commences from the ATG start codon, where +1 is the A of the ATG translation initiation codon (NM_00155.3). Novel variants have been submitted to ARUP *GALT* database.

3.2 | Reverse transcription-PCR (RT-PCR)

The influence of identified novel variants on mRNA splicing was studied by amplification of the segment containing the appropriate exons by RT-PCR. Total mRNA was extracted from whole blood using the PAXgene Blood RNA Kit (Qiagen GmbH, Hilden, Germany) or from fibroblasts. First

strand cDNA was synthesized from isolated mRNA using iScript cDNA Synthesis Kit (Bio-Rad Laboratories Inc.). To confirm the aberrant splicing, cDNA fragments were amplified, using primers covering the area of interest. Amplified products were separated by agarose gel electrophoresis and visualized by gel red staining, GelRed, (Biotium Inc., Fremont, CA). Direct-cycle sequencing of the PCR fragments of interest, was performed with BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA).

3.3 | Bioinformatic analysis

The bioinformatic tools, Polymorphism Phenotyping v2 (PolyPhen-2 v2)²² and Protein Variation Effect Analyzer (Provean v1.1.3),²³ were used for the prediction of the pathogenicity of unpublished nonsynonymous missense variants, and Berkeley Drosophila Genome Project (BDGP)²⁴ was used for synonymous missense variants, splice site variants and intronic variants. Allele frequencies were obtained from Genome Aggregation Database (GnomAD), (<http://gnomad.broadinstitute.org>, accessed 15 May 2019). *GALT* variants were considered novel if not described in HGMD or the ARUP *GALT* database. All variants were checked against the Database of single nucleotide polymorphisms (dbSNP, <https://www.ncbi.nlm.nih.gov/snp>, accessed 22 October 2018).

3.4 | Biochemical analysis

Gal and Gal-1-P were quantified in DBS.²⁵ The detection limit at our laboratory was 0.1 mmol/l (reference value <0.1 mmol/l) for both analytes.

Galactitol was determined using GC-MS according to Allen with modifications.²⁶ The detection limit was 20 μ mol/l (reference value controls, n = 24, <200 mmol/mol creatinine).

GALT activity was measured in erythrocytes with ¹⁴C-labeled Gal-1-P substrate²⁷ after an incubation time of 1 hour (reference value controls, n = 48, 10.4 μ kat/kg Hb, range 7.0–13.1 μ kat/kg Hb). In patients with possible residual activity, the *GALT* activity was measured with incubation times of 1 and 2 hours. To evaluate if prolonged incubation in the Beutler test could be an alternative for the determination of low *GALT* activity in erythrocytes, samples from patients were incubated for 3 and 6 hours in the Beutler test.

4 | RESULTS

4.1 | Molecular analysis

The search for *GALT* variants in the Swedish galactosemia population was initiated in the beginning of the 1990s.²⁰ From a total of nine patients, five were fully characterized. These patients were homozygous for p.(Gln188Arg), as

verified by restriction fragment length polymorphism. The remaining four patients were reinvestigated in this study.

Only data from index cases are included. In addition, two patients with Duarte galactosemia and novel variants are included. The disease-causing variants were detected in all patients and 33 different *GALT* variants were identified (Table 1).

Nineteen patients were homozygous and 17 heterozygous for the most common variant, p.(Gln188Arg). The second most frequent variant was p.(Met142Lys) (c.425T>A), with an allele frequency of 9%. Six patients were compound heterozygous for p.[(Met142Lys)];[(Gln188Arg)], making it the second most common genotype. Only three additional, known variants were found in more than one patient: p.(Leu195Pro) (c.584T>C) (n = 2), p.(Lys285Asn) (n = 3), p.(Arg333Trp) (c.997C>T) (n = 2) and c.508-29delT (n = 2).

A total of 16 novel variants were identified, each in only one patient, except for the two variants: p.(Leu37Arg) (c.110T>G) and p.(Pro109=) (c.327A>G), which were found in two patients (Table 1).

Nineteen patients carry genotypes not described before. Of these, 13 are associated with severe galactosemia, four with a milder disease and two are associated with the D/G disease (Table 2).

4.2 | Prediction of pathogenicity of novel missense variants

All nonsynonymous missense variants were analyzed with the bioinformatic tools PolyPhen-2 v2 and Provean (Table 1). Three novel variants were classified as benign in both tools. The in silico predictions for these variants are in agreement with the phenotypes of the patients. Four novel variants were considered deleterious by both tools and the clinical presentation of the patients supported these predictions.

For the novel variant p.(Ala46Val) (c.137C>T), the predictions were inconclusive. PolyPhen-2 considered the variant “Possibly damaging” while Provean considered it “Neutral.” This variant was detected in a patient who is compound heterozygous for p.(Ala46Val);(Asn314Asp) and diagnosed as D/G. The patient has a *GALT* activity of 25% of normal, and the p.(Ala46Val) is therefore considered a variant associated with severe galactosemia.

4.3 | p.(Arg25Pro)

The variant p.(Arg25Pro) (c.74G>C) was found in combination with p.(Gln188Arg). The screening sample taken at age 5 days showed a *GALT* activity of 3% in the Beutler test and slightly elevated Gal and Gal-1-P: 0.8 and 2.7 mmol/l, respectively. At the time of recall (age 6 days) the patient did not present any clinical symptoms, despite Gal 3.9 mmol/l and Gal-1-P 2.3 mmol/l. Urine galactitol at the

TABLE 1 Variants detected in the Swedish galactosemia population

Systemic name nucleotide change	Predicted effect on protein Trivial name	Number of alleles index patients	GnomAD (%) Sweden ^a	GnomAD (%) Worldwide ^b	dbSNP	Polyphen 2	Provean	References
c.1A>G	p.Met1?	1	(-)	(-)	rs111033639			Calderon et al. ¹⁹
c.74G>C	p.(Arg25Pro)	1	(-)	(-)		Benign	Neutral	This study
c.97C>T	p.(Arg33Cys) ^c	1	(-)	(-)		Probably damaging	Deleterious	This study
c.108G>C	p.(Pro36=)	1	0.0038	0.0024	rs1362198825			This study
c.110T>G	p.(Leu37Arg)	2	0.0038	0.00040	rs774933597	Probably damaging	Deleterious	This study
c.137C>T	p.(Ala46Val)	1	(-)	(-)		Possible damaging	Neutral	This study
c.143G>T	p.(Arg48Leu)	2	(-)	(-)		Probably damaging	Deleterious	This study
c.211C>T	p.(Leu71Phe) ^d	1	0.038	0.0095	rs143994870	Benign	Neutral	This study
c.241G>C	p.(Ala81Pro)	1	0.00	0.00080	rs111033665	Benign	Neutral	This study
c.289_291delAAC	p.(Asn97del)	1	(-)	(-)	rs398123179		Deleterious	Boutron et al. ²⁸
c.327A>G	p.(Pro109=) ^e	3	(-)	(-)				This study
c.377+1G>A	p.?	2	0.00	0.00080	rs1111033681			HGMD Professional 2018:3.
c.425T>A	p.(Met142Lys)	10	0.015	0.0020	rs111033695	Probably damaging	Deleterious	Reichardt et al. ¹⁵
c.478_480delGAG	p.(Gln160del)	1	(-)	(-)			Deleterious	This study
c.508-29delT	p.(Gln169_Ile170insTer20)	4	(-)	(-)	rs111033711			Bosch et al. ²⁹
c.508-2_509delAGAT	p.(Gln169_Ile170ins21)	1	(-)	(-)				This study
c.563A>G	p.(Gln188Arg)	55	0.21	0.15	rs75391579	Probably damaging	Deleterious	Reichardt et al. ¹⁵
c.584T>C	p.(Leu195Pro)	2	0.023	0.0072	rs111033728	Possible damaging	Deleterious	Reichardt et al. ¹⁵
c.594delT	p.(Ile198MetfsTer21)	2	(-)	(-)				This study
c.602G>A	p.(Arg201His)	1	0.0038	0.0025	rs111033735	Benign	Neutral	Elsas et al. ³⁰
c.748C>T	p.(Pro250Ser)	2	(-)	(-)		Probably damaging	Deleterious	This study
c.772C>T	p.(Arg258Cys)	1	0.011	0.0032	rs368166217	Probably damaging	Deleterious	Tyfield et al. ³¹
c.775C>T	p.(Arg259Trp)	2	0.00	0.0012	rs786204763	Probably damaging	Deleterious	Shin-Bühning et al. ²⁷
c.815G>A	p.(Arg272His)	1	0.00	0.00080	rs111033831	Probably damaging	Deleterious	Boutron et al. ²⁸
c.820+5G>A	p.(?)	1	0.0038	0.00040	rs1473388542			This study
c.855G>T	p.(Lys285Asn)	5	0.011	0.013	rs111033773	Probably damaging	Deleterious	Leslie et al. ³²
c.896G>A	p.(Gly299Asp)	2	(-)	(-)		Probably damaging	Deleterious	This study

(Continues)

TABLE 1 (Continued)

Systemic name nucleotide change	Predicted effect on protein Trivial name	Number of alleles in index patients	GnomAD (%) Sweden ^a	GnomAD (%) Worldwide ^b	dbSNP	Polyphen 2	Provean	References
c.940A>G	p.(Asn314Asp)	2	8.5	9.0	rs2070074	Benign	Neutral	Elsas et al. ³³
c.958G>A	p.(Ala320Thr) ^c	1	0.00	0.0012	rs111033795	Probably damaging	Deleterious	Elsas et al. ³⁰
c.974C>T	p.(Pro325Leu)	1	0.00	0.0014	rs111033794	Probably damaging	Deleterious	Greber-Platzner et al. ³⁴
c.997C>T	p.(Arg333Trp)	1	0.0038	0.0028	rs111033800	Probably damaging	Deleterious	Reichardt et al. ¹⁵
c.1001_1002delinsT	p.(Lys334IlefsTer25)	1	(-)	(-)				This study
c.1059+56C>T	p.(Gln353_Ala354ins18)	1	(-)	(-)	rs111033821			Wadelius et al. ²⁰

Note: Novel variants are depicted in bold. GnomAD v2.1.1, db SNP Database of single nucleotide polymorphisms. Position according to the reference sequence NM_00155.3.

Abbreviation: -, not found in GnomAD.

^aAllele number = 26 130.

^bAllele number = 251 000-283 000.

^cIn *cis* with p.(Asn314Asp).

^dIn *cis* with p.(Glu160del).

^eHomozygous patient.

time of recall was 1940 mmol/mol creatinine, which is elevated but lower than usually seen in patients with classical galactosemia. Quantitative determination of GALT activity in erythrocytes with ¹⁴C-labeled Gal-1-P substrate showed an activity of 3% of normal (Table 3). The patient was classified as having a milder form of classical galactosemia and had normal secretion of galactitol in urine during dietary treatment.

4.4 | p.(Pro36=)

The synonymous variant p.(Pro36=) (c.108G>C) was detected in combination with p.(Gln188Arg). The patient was re-called with a presumptive classical galactosemia. The patient had a residual activity of 9% in the Beutler test and the concentrations of Gal and Gal-1-P were 7.8 and 0.5 mmol/l, respectively. Galactitol in urine was 9600 mmol/mol creatinine. The second DBS sample showed a GALT activity of 12% in the Beutler test. A prolonged incubation time in the Beutler test from 3 to 6 hours showed a proportional increase of fluorescence values over time. An increase in fluorescence is not seen in patients with two null variants (Figure 1A).

In silico analysis predicted the synonymous variant p.(Pro36=) to create a new acceptor splice site 32 base pairs upstream of the authentic site and seven base pairs from the variant. The splicing pattern for p.(Pro36=) was investigated in mRNA isolated from leukocytes. The cDNA transcript, when visualized on a 3% agarose gel after electrophoresis, showed two different bands—one of normal and one of shorter length (Figure 1B). Sanger sequencing of the shorter PCR product confirmed the in silico prediction. The p.(Pro36=) variant is classified as mild and the patient had normal secretion of galactitol in urine during dietary treatment.

4.5 | p.(Ala81Pro)

The variant p.(Ala81Pro) (c.241G>C) was found together with p.(Gln188Arg). The screening sample taken at age 3 days showed a GALT activity of 8% in the Beutler test and Gal and Gal-1-P levels were 0.3 and 1.9 mmol/l, respectively. The same low activity in the Beutler test was found in the second DBS, with Gal and Gal-1-P levels of 0.6 and 1.4 mmol/l. Galactitol in urine was 1050 mmol/mol creatinine. GALT activity in erythrocytes with ¹⁴C-labeled Gal-1-P was 10% of normal. A longer incubation in the Beutler test from 3 to 6 hours showed a proportional increase of fluorescence over time, indicating that the patient has an ability to metabolize Gal-1-P to some extent, but at a slower rate than healthy controls (Figure 1A). The p.(Ala81Pro) variant is classified as mild and the patient had normal secretion of galactitol in urine without dietary treatment.

TABLE 2 Genotypes in patients with novel variants in the *GALT* gene

Allele 1		Allele 2		Allele 2		Age at first sample days	BIA ^a /Beutler test	Gal (mmol/l)	Galactitol (mmol/mol) creatinine	Severity
Systemic name Nucleotide change	Predicted effect on protein	Systemic name Nucleotide change	Predicted effect on protein	Predicted effect on protein						
c.74G>C	p.(Arg25Pro)	c.563A>G	p.(Gln188Arg)	p.(Gln188Arg)	5	3%	0.8	2.7	1900	Mild
c.[97C>T;940A>G]	p.[(Arg33Cys);(Asn314Asp)]	c.563A>G	p.(Gln188Arg)	p.(Gln188Arg)	3	6%	14.1	6.7	2700	Classical
c.108G>C	p.(Pro36=)	c.563A>G	p.(Gln188Arg)	p.(Gln188Arg)	8	9%	7.8	0.5	9600	Mild
c.110T>G	p.(Leu37Arg)	c.855G>T	p.(Lys285Asn)	p.(Lys285Asn)	4	8%	9.3	2.7	13 000	Classical
c.110T>G ^b	p.(Leu37Arg)	c.425T>A	p.(Met142Lys)	p.(Met142Lys)	n.a.	BIA	n.a.	n.a.	n.a.	Classical
c.137C>T ^b	p.(Ala46Val)	c.940A>G	p.(Asn314Asp)	p.(Asn314Asp)	n.a.	n.a.	n.a.	n.a.	n.a.	Duarte ^f
c.143G>T	p.(Arg48Leu)	c.143G>T	p.(Arg48Leu)	p.(Arg48Leu)	2 months ^c	12%	<0.1	0.2	—	Classical
c.241G>C	p.(Ala81Pro)	c.563A>G	p.(Gln188Arg)	p.(Gln188Arg)	2	8%	<0.1	<0.1	n.d	Mild
c.327A>G	p.(Pro109=)	c.327A>G	p.(Pro109=)	p.(Pro109=)	8	7%	15.8	4.1	17 700	Classical
c.327A>G	p.(Pro109=)	c.940A>G	p.(Asn314Asp)	p.(Asn314Asp)	5	7%	<0.1	0.93	n.a.	Duarte ^f
c.[478_480del;211C>T]	p.[(Gln160del);(Leu71Phe)]	c.1059+56C>T	p.(Gln353_Ala354ins18)	p.(Gln353_Ala354ins18)	n.a.	BIA	n.a.	n.a.	n.a.	Classical
c.508-2_509del/AGAT	p.(Gln169_Ile170ins21)	c.563A>G	p.(Gln188Arg)	p.(Gln188Arg)	5	4%	5.8	1.5	7100	Classical
c.594delT ^d	p.(Ile198MetfsTer21)	c.594delT	p.(Ile198MetfsTer21)	p.(Ile198MetfsTer21)	Not screened	3%	—	—	—	Classical
c.748C>T ^b	p.(Pro250Ser)	c.748C>T	p.(Pro250Ser)	p.(Pro250Ser)	Not screened	—	—	—	—	Classical
c.820+5G>A	p.(?)	c.563A>G	p.(Gln188Arg)	p.(Gln188Arg)	n.a.	BIA	1.4	4.1	n.a.	Classical
c.896G>A	p.(Gly299Asp)	c.896G>A	p.(Gly299Asp)	p.(Gly299Asp)	3	10%	7.0	1.0	n.a.	Classical
c.1001delinsT	p.(?)	c.563A>G	p.(Gln188Arg)	p.(Gln188Arg)	n.a.	3%	10.3	5.5	n.a.	Classical
c.[940A>G;958G>A]	p.[(Asn314Asp);(Ala320Thr)]	c.563A>G	p.(Gln188Arg)	p.(Gln188Arg)	2	6%	2.1 ^e	2.5 ^e	—	Classical

Note: Novel variants depicted in bold. Position according to reference sequence NM_001553.

^aBIA bacterial inhibition assay.

^bNot verified in parents.

^cAnalyzed at arrival in Sweden.

^dDiagnosed in home country and on treatment.

^eAnalyzed at age 4 days (second sampling).

^fPatients with Duarte galactosemia on nonrestricted diet.

4.6 | p.(Pro109=)

The synonymous variant p.(Pro109=) (c.327A>G), was found in a homozygous state in one patient with classical galactosemia and in compound heterozygous state together with p.(Asn314Asp) in a D/G patient.

The patient, homozygous for this variant, had a GALT activity of 7% in the Beutler test and Gal and Gal-1-P values of 15.8 and 4.1 mmol/l, respectively. Urine galactitol was markedly elevated, 17 700 mmol/mol creatinine. Quantitative measurement of GALT activity in erythrocytes with ¹⁴C-labeled Gal-1-P showed an activity of 0.8% and 0.9% of normal after 1 and 2 hours of incubation, respectively (Table 3).

The second patient, who was compound heterozygous for p.[(Pro109=)];[(Asn314Asp)], also had a GALT activity of 7% in the Beutler test. The concentrations of Gal and Gal-1-P were <0.1 and 0.93 mmol/l, respectively. The patient

TABLE 3 Erythrocyte GALT activity

Genotype	1 hour	2 hour
	μkat/kg Hb (%)	μkat/kg Hb (%)
p.[=];[=]	9.8 (100)	18.0 (100)
p.[(Arg25Pro)];[(Gln188Arg)]	0.26 (3.0)	0.47 (2.6)
p.[(Pro109=)];[(Pro109=)]	0.08 (0.8)	0.17 (0.9)
p.[(Gln188Arg)];[(Arg333Trp)]	0.05 (0.5)	-0.05 (0)

Note: GALT activity measured in erythrocytes at time point 1 and 2 hours in two patients with residual activity.

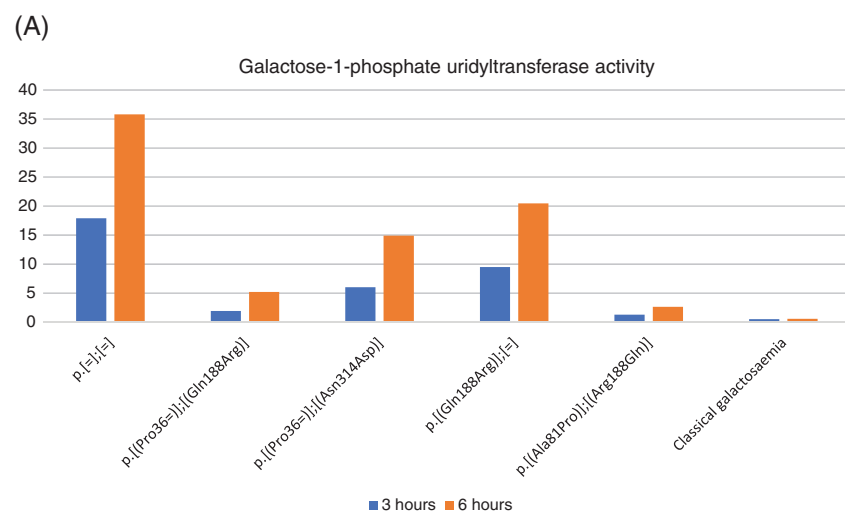


FIGURE 1 (A) Relative increase in fluorescence seen over time for the novel variants p.(Pro36=) and p.(Ala81Pro). Both these variants are in compound heterozygous state with p.(Gln188Arg) and analyzed with the Beutler assay. Fluorescence was measured at the time points 3 and 6 hours. Samples from a healthy individual, p.[=];[=] and a patient who is compound heterozygous for p.[(Gln188Arg)];[=], 50% GALT-activity, were used as controls. (B) cDNA transcript after RT-PCR of the novel synonymous variant p.(Pro36=). The transcripts were visualized on a 3% agarose gel resulting in an additional 32 bp shorter product. Lane 1, size standard, lane 2, patient compound heterozygous for p.[(Gln188Arg)];[=], lane 3, patient compound heterozygous for p.[(Pro36=)];[(Asn314Asp)], lane 4, control (wild type)

has always been on an unrestricted diet and is classified as having D/G.

According to in silico analysis, the synonymous variant p.(Pro109=) is predicted to weaken the authentic donor site in exon 3, a decline in BDGP score from 0.90 to 0.36. The splicing pattern for p.(Pro109=) was investigated in mRNA isolated from fibroblasts. The cDNA transcript, when visualized on a 3% agarose gel, showed four different bands which were subjected to Sanger sequencing (Figure 2A). The detection of a faint band of normal length probably explains

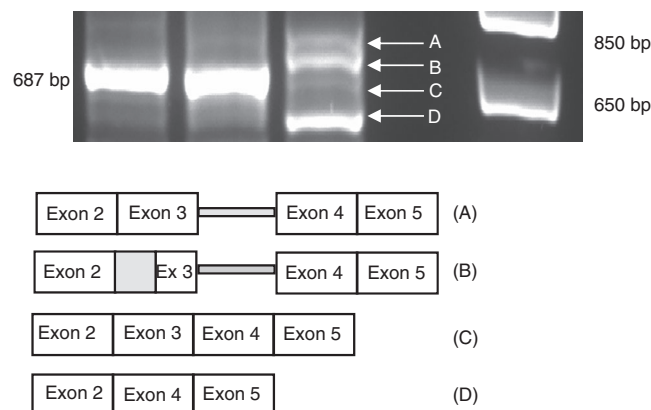


FIGURE 2 cDNA transcript after RT-PCR of the novel synonymous variant p.(Pro109=). The transcripts were visualized on a 3% agarose gel. Lane 1 and 2, wild type, lane 3 patient homozygous for p.(Pro109=), lane 4 size standard. (A) Insertion of intron 3, (B) skipping of 49 bp of exon 3 and insertion of intron 3, (C) wild type, (D) skipping of exon 3

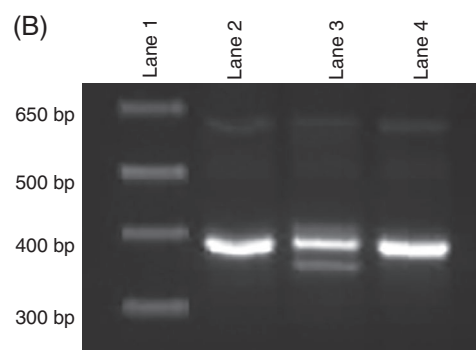


TABLE 4 Mendelian inheritance in a family with four *GALT* alleles

	Allele 1	Allele 2	GALT activity % of normal in erythrocytes
Patient	p.[(Leu71Phe);(Glu160del)]	p.(Gln353_Ala354ins18)	0.1
Mother	p.[=]	p.(Gln353_Ala354ins18)	50
Father	p.(Leu71Phe)	p.(Asn314Asp)	76
Sibling	p.(Asn314Asp)	p.(Gln353_Ala354ins18)	29

the presence of a low residual activity of GALT in erythrocytes. The p.(Pro109=) variant is classified as a less severe variant of classical galactosemia.

4.7 | p.(Glu160del)

The novel in-frame variant, p.(Glu160del) (c.478_480delGAG), was found in *cis* with p.(Leu71Phe) (c.211C>T). The patient was previously found to carry a cryptic splice site variant p.(Gln353_Ala354ins18) (c.1059+56C>T) in intron 10 on one allele.²⁰ The cryptic splice site variant leads to an insertion of 18 amino acid as determined at the cDNA level. Mendelian inheritance estimation and GALT activity measurements were performed in the family (Table 4). The result of the measurements of GALT activity was inconclusive until sequencing analysis of the family was performed. Genetic analysis revealed, that the father was compound heterozygous for p.(Leu71Phe) and p.(Asn314Asp), the latter being the Duarte variant. The sibling was compound heterozygous for the D/G genotype, p. [(Asn314Asp)];[(Gln353_Ala354ins18)]. The mother was carrier of the p.(Gln353_Ala354ins18). The p.(Glu160del) variant was not found in the father or mother indicating that it was a *de novo* mutation. According to the patient's phenotype, the in frame variant p.(Glu160del), is most probably associated with classical galactosemia.

Based on the father's genotype p.[(Leu71Phe)];[(Asn314Asp)] and GALT activity of 76% of normal in erythrocytes, the variant p.(Leu71Phe) is classified as benign. To our knowledge the variant p.(Leu71Phe) has not been described in patients with galactosemia (Table 1).

4.8 | c.508-29delT and c.508-2_509delAGAT

The variant c.508-29delT has previously been described in a patient of Somali ancestry with classical galactosemia.²⁹ The authors speculated that the variant may affect a branch point sequence in the *GALT* gene, resulting in a disruption of the authentic splice site. In our cohort, the variant c.508-29delT was found in a homozygous state in two patients of Somali ancestry as well.

The novel variant c.508-2_509delAGAT deletes the last two nucleotides in intron 5 and the two first nucleotides in exon 6, compromising the authentic 3'acceptor site. This

variant was detected together with p.(Gln188Arg) in a patient with classical galactosemia.

In silico analysis with BGD³⁰ of each of these variants predicted the loss of the authentic 3'acceptor splice (BDGP score 0.93), while creating a cryptic splice site (BDGP score of 0.98).

We investigated the aberrant splicing in mRNA from fibroblasts of a patient homozygous for c.508-29delT and from leukocytes of the patient heterozygous for the c.508-2_509delAGAT variant. Both variants activated the predicted cryptic splice site, between nucleotides -68 and -67 upstream of c.508 (Supporting Information, Figures S1 and S2).

4.9 | Variants in *cis* with p.(Asn314Asp)

Due to the high allele frequency of the Duarte variant, it is not uncommon to find the variant in *cis* with another galactosemia variant. In our study, the novel variant p.(Arg33Cys) (c.97C>T) and the earlier described variant p.(Ala320Thr) (c.958G>A)³⁰ were found in *cis* with p.(Asn314Asp). Both patients carry the common p.(Gln188Arg) variant on the second allele and have the classical phenotype.

5 | DISCUSSION

This is the first complete genetic study of patients with GALT deficiency in a Scandinavian country. All known patients, except one, are included in the survey. The birth incidence of GALT deficiency in Sweden is approximately 1/100 000, which is the same as in France and Italy, but lower than the reported incidence of 1/30 000-40 000 for most other European countries.^{28,35,36}

Thirty-three variants were detected in the Swedish galactosemia population, and of these 16 are unique for our cohort. Fifteen are not found in HGMD or ARUP *GALT* database and one has previously been published in 1993.²⁰ Only a few variants are frequent, which agrees with reports from other countries.^{30,31,37,38} The most common variant, among Swedish patients with classical galactosemia is p.(Gln188Arg) with an allele frequency of 51%.

The missense variant, p.(Met142Lys), was one of the first variants identified to cause galactosemia.¹⁶ Although it is well described, this is the first time to our knowledge, that p.(Met142Lys) is reported as the second most common variant

in a population. Eight of the patients carrying this variant are of Swedish or Norwegian origin, while the ninth is from Lithuania.

Two novel variants were found in more than one patient, the variants p.(Leu37Arg) and p.(Pro109=). The two patients heterozygous for p.(Leu37Arg) carry the variants p.(Met142Lys) and p.(Lys285Asn) on the second allele, respectively. Both presented with severe clinical symptoms before the initiation of treatment.

The Duarte variant, p.(Asn314Asp), is found in all populations, albeit with different frequencies. According to the database GnomAD the highest allele frequency is found in South Asia (18%), as compared to an allele frequency of 9.4% in Europe (non-Finnish). The lowest frequency is found in East Asia (0.9%). At least nine missense variants and four nonsense variants have previously been described in *cis* with the Duarte variant (Supplemental Table S3).^{28,30,31,39-42}

The two complex variants p.(Arg33Cys;Asn314Asp) and p.(Asn314Asp;Ala320Thr) in our population increase this group to 15. These results demonstrate the importance of sequencing the whole *GALT* gene and not only a panel including common variants. If only a panel is analyzed and p.(Asn314Asp) is included, there is a risk of misdiagnosis, as the p.(Asn314Asp) variant frequently occurs in *cis* with more rare variants.

Occasionally, patients with D/G are recalled because of a *GALT* activity <15% in the Beutler test. The patient who is compound heterozygous for p.[(Ala81Pro)];[(Gln188Arg)] was recalled due to a residual activity of 8%, which was the same in the repeat sample, while Gal-1-P was only slightly elevated. Genotyping detected the novel variant p.(Ala81Pro). A disease-causing variant in the same codon p.(Ala81Thr) (c.241G>A) has been described.³² To assist estimation of the pathogenicity of p.(Ala81Pro), the variant was evaluated in PolyPhen-2, with the result “Benign,” in contrast to p.(Ala81Thr), which was denoted “Probably damaging.” The variant p.(Ala81Pro) reduces *GALT* activity in the Beutler test to the same extent as in classical galactosemia patients. Blood samples from the patient with this mutation in combination with p.(Gln188Arg) still have the ability to metabolize Gal-1-P, although at a slower rate than samples from healthy controls. This was seen when measuring *GALT* activity quantitatively in erythrocytes as well as in a prolonged Beutler test. We consequently estimate that the variant p.(Ala81Pro) results in a severity equal to Duarte galactosemia. The patient has been on a normal diet from 1 year of age, is close to 10 years old and has had a normal development. All the patients with mild galactosemia have had a normal secretion of galactitol after the newborn period.

The patient with the de novo mutation described here illustrates the importance of sequencing the complete *GALT* gene in all family members when the results of the measurement of *GALT* activity are inconclusive.

5.1 | Impact of synonymous variants

Synonymous variants in the *GALT* gene are common. The ARUP *GALT* database and Exome Aggregation Consortium (ExAC) (<http://exac.broadinstitute.org>, accessed 22 October 2018) contain 12 and 44 unique synonymous variants, respectively. An additional seven variants are found in both databases. Fifteen of the synonymous variants in the ARUP *GALT* database are described as benign, whereas four are of uncertain severity. After *in-silico* analysis of all 63 variants in BDGP, only six variants were predicted to either abolish or weaken an existing splice site or create a new splice site. Included in the six synonymous variants, predicted to affect splicing, are the three variants proven to be pathogenic: p.(Pro36=), p.(Pro109=) and the earlier presumably benign synonymous variant, p.(Gly338=) (c.1014C>G). The latter has been shown to be disease-causing by activating an alternative splice donor site, resulting in the exclusion of 46 nucleotides at the end of exon 10.²⁸ The two synonymous variants described in this article both result in reduced *GALT* activity. The variant p.(Pro36=) activates a new acceptor splice site, excluding 32 nucleotides at the beginning of exon 2, giving a shorter protein. Amplification of *GALT* cDNA carrying the p.(Pro109=) variant revealed the presence of multiple fragments from RT-PCR (Figure 2A, B). This strongly suggests that the p.(Pro109=) allele in this patient generates a normal transcript, two longer and one shorter transcript, supporting the idea that all four 5' splicing sites, cryptic and normal are used.

Splice variants that generate both normal and abnormal transcripts from the same allele are referred to as leaky. The relative amounts of normal and aberrant splice products generated are important; the higher the levels of normal transcript, the milder the phenotype. The presence of some normal transcript is probably generating the residual *GALT* activity of approximately 1%, which is supported by the 1- and 2-hour incubation of erythrocytes with ¹⁴C-labeled Gal-1-P and the somewhat milder phenotype of the patient. Our results, together with the results of Bourton et al 2012, strengthen the importance to evaluate the consequences of synonymous variants at the mRNA level, especially if no other variants in the *GALT* gene are detected.²⁸

Classical galactosemia is a potentially progressive disease.⁴ Our experience from the three patients described in this paper with some residual activity is that a *GALT* activity of 1% is not enough to prevent severe symptoms before diagnosis or to prevent long term complications when the patient is on Gal restricted diet.

Sweden moved the sampling time from as early as possible after 72 hours, to as early as possible after 48 hours of age in 2008. An important advantage of the earlier sampling time is that patients are recalled at a younger age. This

increases the chance of diagnosis and commencement of treatment, before the development of life-threatening symptoms.

6 | CONCLUSIONS

Classical galactosemia is a rare disease in Sweden affecting 1/100 000 newborn infants. Like in other countries only a few variants in the *GALT* gene are frequent in the cohort. In this study 16 of the 33 variants are novel including a probable de novo variant.

The variants p.(Pro36=) and p.(Pro109=) are two new synonymous variants in the *GALT* gene proven to be pathogenic. This demonstrates the importance of evaluating the consequences of synonymous variants in the *GALT* gene at the cDNA level.

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COMPLIANCE WITH ETHICS GUIDELINES

CONFLICT OF INTEREST

Annika Ohlsson, Mary Hunt, Anna Wedell and Ulrika von Döbeln declare that they have no conflict of interest.

INFORMED CONSENT

All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2000.

According to the ethical permission, informed consent was not requested.

Ethical permission: Genetic investigation of inborn errors of metabolism, approved by Regional Ethical Committee of Stockholm, 2008/351-31.

CONTRIBUTION OF INDIVIDUAL AUTHORS

Annika Ohlsson planned, designed and interpreted the data and wrote the draft of the manuscript.

Ulrika von Döbeln planned, designed and interpreted the data and revised the manuscript.

Mary Hunt planned and designed some of the experiments and revised the manuscript.

Anna Wedell interpreted data and revised the manuscript. All authors agree to submission of the manuscript.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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