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REVIEW

Galactose-1-phosphate uridyltransferase deficiency: A literature review of the putative mechanisms of short and long-term complications and allelic variants

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Galactosemia type 1 is an autosomal recessive disorder of galactose metabolism, determined by a deficiency in the enzyme galactose-1-phosphate uridyltransferase (GALT). GALT deficiency is classified as severe or variant depending on biochemical phenotype, genotype and potential to develop acute and long-term complications. Neonatal symptoms usually resolve after galactose-restricted diet; however, some patients, despite the diet, can develop long-term complications, in particular when the GALT enzyme activity results absent or severely decreased. The mechanisms of acute and long-term complications are still discussed and several hypotheses are presented in the literature like enzymatic inhibition, osmotic stress, endoplasmic reticulum stress, oxidative stress, defects of glycosylation or epigenetic modification. This review summarizes the current knowledge of galactosemia, in particular the putative mechanisms of neonatal and long-term complications and the molecular genetics of GALT deficiency.

KEYWORDS

galactosemia, genotype, neonatal and long-term complications, variants

1 | INTRODUCTION

Galactosemia is an autosomal recessive disorder of galactose metabolism. Galactose is derived mainly from the lactose introduced into the diet and successively metabolized through the following steps: (1) β-D-galactose is epimerized to α-D-galactose by galactose mutarotase; (2) α-D-galactose is phosphorylated to galactose-1-phosphate (Gal-1-P) by the galactokinase (GALK); (3) Gal-1-P is metabolized by the galactose-1-phosphate uridyltransferase (GALT), through a double displacement mechanism: GALT transfers uridine monophosphate from uridine diphosphate glucose (UDP-glc) to Gal-1-P with consequent production of uridine diphosphate galactose (UDP-gal) and glucose-1-phosphate (Glc-1-P); and (4) UDP-gal is converted to UDP-glc by the galactose epimerase (GALE).[1] UDP-gal is a galactose donor for the synthesis of glycoproteins and glycolipids.[1,2]

Three different forms of galactosemia are determined by the enzyme deficiencies of GALT (Galactosemia type 1), GALK (Galactosemia type 2), or GALE (Galactosemia type 3).[1] GALT deficiency (OMIM 230400) is the most frequent form, with a prevalence of about 1 in 30 000 to 60 000 live births in the world population,[3–5] and 1 in 47 000 in the Caucasians.[6] GALT deficiency is classified as severe or variant depending on biochemical phenotype, genotype and potential to develop acute and long-term complications.[7] In particular, the severe phenotype is associated with neonatal symptoms and long-term complications, whereas clinical variant galactosemia shows less severe neonatal symptoms and absent or mild long-term complications with early dietary treatment. The variant form includes

Abbreviations: CDG, congenital disorders of glycosylation; D, Duarte; D1, Duarte-1; D2, Duarte-2; ER, endoplasmic reticulum; FSH, follicle-stimulating hormone; Gal, galactose; Gal-1-P, galactose-1-phosphate; GALE, UDP-galactose 4-epimerase; GALK, galactokinase; GALT, Galactose-1-Phosphate Uridyltransferase; Glc-1-P, glucose-1-phosphate; LEC, lens epithelial cells; POI, primary ovary insufficiency; UDP-gal, uridine diphosphate galactose; UDP-glc, uridine diphosphate glucose; UGP, UDP-glucose pyrophosphorylase; UPR, unfolded protein response

Duarte (D) galactosemia, which is approximately 10 times more frequent than the classic form, about 1:4000 live births in the Caucasian population.[8]

A high percentage of galactosemic subjects is now detected through newborn screening programs that now operate in many countries.[9–13] Deaths related to galactosemia in the neonatal period decreased significantly after the introduction of newborn screening,[10] but a high percentage of patients continue to exhibit clinical symptoms in the first few days of life and long-term complications.[14–16]

2 | NEONATAL AND LONG-TERM COMPLICATIONS OF GALACTOSEMIA

Newborns with galactosemia can present symptoms after milk introduction, including failure to thrive, poor feeding, vomiting, diarrhea, or other less common ones such as lethargy, hepatomegaly, bleeding diathesis, renal tubule dysfunction, *pseudotumor cerebri* or cataract. Without dietary treatment, these patients can also exhibit a progressive liver or kidney failure, sepsis or shock with consequent death in the neonatal life. An early galactose-restricted diet usually resolves these acute symptoms.[7,17,18] However, even with dietary treatment, some patients develop long-term complications such as neurological disorders or ovarian failure. The high levels of Gal-1-P are caused by severely reduced or absent GALT activity,[19,20] which in turns inhibits GALK and leads to an accumulation of galactose. Aldose reductase converts then the excess of galactose into galactitol, which accumulates because it is not further catabolised.[21,22] Gal-1-P accumulation might depend upon 1 or more of the following factors: (1) the level of residual GALT enzyme activity: in fact, a study on patient-derived alleles expressed in yeast or *Escherichia coli* showed that GALT activity of <1% of wild-type levels leads to accumulation of Gal-1-P and significant loss of UDP-gal, whereas yeast with GALT activity of 1%-5% showed only transient Gal-1-P accumulation, and finally in the presence of GALT activity >10%, no Gal-1-P accumulates[23]. (2) The capacity of a tissue to synthesize the GALT enzyme. For example, in case of the variant galactosemia frequently observed in African-Americans, the GALT activity in the liver is about 10%, whereas in the erythrocytes it ranges from <1% to about 4% of wild-type levels.[24] The residual enzyme activity in the liver determined the absence of long-term complications in these patients. One hypothesis to explain the discrepancy between erythrocytes and liver GALT activity is that the African-American allelic variant exhibits an enhanced degradation rate of the GALT protein that is not synthesized by erythrocytes.[25] (3) Individual variability, in particular renal efficiency and residual capacity to metabolize galactose by alternative pathways.[26] However, the role of Gal-1-P in the pathogenesis of galactosemia is still questioned. Experiments in GALT-knockout yeast *Saccharomyces cerevisiae* and in human cell lines have showed that Gal-1-P accumulation is toxic because it reduces the growth.[19,20,27] Moreover, in GALK patients where Gal-1-P does not accumulate, the absence of long-term complications supported the hypothesis that Gal-1-P is toxic in galactosemic patients.[28] On the other hand, a recent study showed that the short- and long-term outcomes occur independently from Gal-1-P using a double mutant GALT/GALK in *Drosophila*.[29] In Figure 1, we report the principal mechanisms suggested for short- and long-term complications.

3 | PUTATIVE MECHANISMS OF NEONATAL COMPLICATIONS

The causes for the insurgence of neonatal complications are still debated; in particular, an unresolved question is whether the accumulated metabolites are deleterious for galactosemic patients. Since high levels of galactose and its metabolites, Gal-1-P and galactitol, are found in the liver, lungs and amniotic fluid of affected fetuses, and because cases of cataract and liver dysfunction have been reported already in prenatal life,[30,31] some authors suggested that prenatal galactose intoxication can lead to neonatal consequences.[21,32,33]

3.1 | Hypoglycemia

Experiments in humans showed that galactose intake increases insulin and glucose levels in the blood suggesting that the hypoglycemia present in some galactosemic newborns is related to the increase in insulin secretion.[34] However, other findings support the hypothesis that hypoglycemia could be related to glycogenolysis defects due to inhibition of glycogen phosphorylase and phosphoglucomutase by Gal-1-P.[35] In fact, insulin and glucose levels in the blood increase immediately after galactose ingestion in humans, and when galactose returns to basal levels, glucose concentration is still high, suggesting a release of glucose by glycogenolysis.[35,36] This is also confirmed by kinetic studies in vitro on isolated enzymes showing that Gal-1-P inhibits glycogen phosphorylase and phosphoglucomutase.[19]

3.2 | Lethargy and irritability

Hypoglycemia and/or the inhibition of glucose uptake by high level of glucose in the brain is suggested to cause lethargy and irritability.[37,38]

3.3 | Liver dysfunction

The inhibition of glycogen phosphorylas and phosphoglucomutase in the liver probably causes glycogen accumulation in hepatic cells leading to hepatomegaly and increase of liver enzymes.[34] However, in untreated galactosemic patients macrovesicular steatosis is commonly reported, with evolution to fibrosis and cirrhosis, instead of glycogen accumulation.[39,40] In more recent studies, young rats fed with galactose exhibit similar histopathological modification in the liver and suggest that oxidative stress has an important role in liver dysfunction[41,42].

3.4 | Kidney dysfunction

High levels of Gal-1-P determine a competitive inhibition of amino acid transport in the kidney and consequent aminoaciduria (tubular kidney dysfunction) as showed by experiments in vitro.[43,44] However, other evidences support the role of altered glycosylation in the renal injury. The investigators showed altered N-glycosylation on urinary

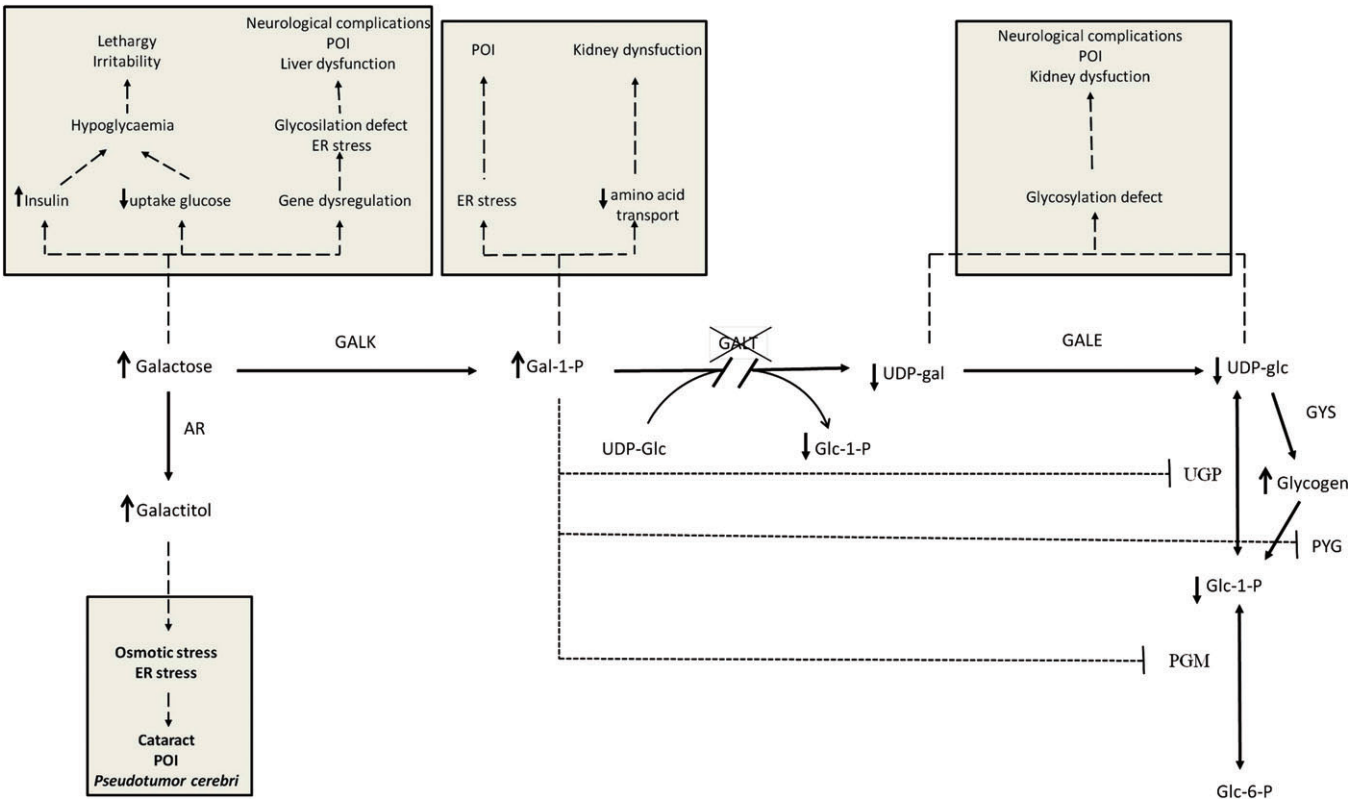

FIGURE 1 Putative mechanisms of neonatal or long-term complications in galactose-1-phosphate uridyltransferase (GALT) deficiency. GALT deficiency determines an increase of galactose, Gal-1-P, galactitol and a decrease of uridine diphosphate galactose (UDP-gal), uridine diphosphate glucose (UDP-glc), Glc-1-P. Gal-1-P can inhibit UDP-glucose pyrophosphorylase (UGP), glycogen phosphorylase (PYG) or phosphoglucomutase (PGM). The broken lines with blunt ends indicate an inhibitory effect. The proposed mechanisms of neonatal or long-term complications are shown by broken arrows in the gray boxes

exosomal membranes, in particular an increase of complex-type N-glycosylation due to an increase of glycoproteins involved in extracellular matrix and basement membrane assembly, suggested an alteration of these 2 mechanisms.[45]

3.5 | Pseudotumor cerebri and cataracts

High levels of galactitol are suggested to be involved in the pathogenesis of acute complications like *pseudotumor cerebri* or cataracts.[28,46] The investigators show that high levels of galactitol are reported in the amniotic fluid of affected fetuses[21,32,33] and in the urine of adult galactosemic patients.[47] Moreover, galactitol accumulation is found in *post-mortem* brain analysis and magnetic resonance imaging in galactosemic children.[48–50] The role of galactitol in the pathogenesis of galactosemia derived also by the similar clinical findings in GALK deficiency, which is characterized by galactitol and galactonate, but not Gal-1-P accumulation.[28] Finally, aldose reductase inhibitors, which block the conversion of galactose in galactitol, block the progression of galactose-induced cataracts in animal models.[51,52] The mechanism hypothesized for the damage induced by galactitol is the osmotic stress. In fact, experiments in rats fed with high doses of galactose showed that galactitol, which does not cross the cell membranes easily, accumulates in the intracellular compartment with the consequent transport of water into the cell. This causes the lens to swell and induces apoptosis of the lens epithelial cells (LECs) and in turn cataract.[46] However, studies on cultured LECs have showed that the osmotic stress induces endoplasmic reticulum (ER) stress that in turn activates the unfolded protein response (UPR). UPR increases production of reactive oxygen species that decrease the level of glutathione, an antioxidant suggesting a role of oxidative stress in the pathogenesis of cataract.[53] Because early dietary treatment led to the complete resolution of cataracts in the acute phase, it has been suggested that the cells lost to apoptosis are replaced with new, functional LECs. It has been proposed that osmotic stress is the main contributor to cataract formation in the acute phase, while oxidative stress is the link between formation and progression.[54,55]

4 | PUTATIVE MECHANISMS OF LONG-TERM COMPLICATIONS

Despite dietary treatment, patients with classic galactosemia often develop long-term complications, in particular neurological alterations and ovarian failure. One of the proposed mechanisms, albeit debated, is defective glycosylation. Some investigators show alterations in N- and O-linked glycosylation in some newborns that normalize or near-normalize following galactose-restricted diet, while few modification in the same pattern were seen in young or adult galactosemic subjects on diet treatment.[56] However, other studies showed N-glycan assembly and processing of transferrin with increase of truncated glycans deficient in galactose and sialic acid, and alteration in

fucosylation in untreated patients, that normalized only in some patients following galactose-restricted diet.[57–59]

Defective glycosylation might depend upon the low levels of UDP-gal and UDP-glc that are carbohydrate donors for numerous galacto-/glycoproteins and galacto-/glycolipids. The following causes were suggested: (1) low levels of residual GALT activity; (2) accumulation of Gal-1-P, which inhibits competitively UDP-glucose pyrophosphorylase that converts Glc-1-P into UDP-glc, as shown in experiments in fibroblasts derived from galactosemic patient[19]; (3) deprivation of galactose due to an over-restrictive diet; and (4) epigenetic modifications, in particular it has been hypothesized that galactose intoxication can induce the dysregulation of several genes involved in pathways that include N- and O-glycan biosynthesis, inositol and inflammatory pathways.[57] Moreover, some of the dysregulated genes are also involved in the pathogenesis of congenital disorders of glycosylation (CDG) type I and II.[60] However, the mechanism by which the galactose intoxication determines this epigenetic modification is unknown.

Low levels of glycoproteins and glycolipids are responsible for abnormal myelin synthesis, protein misfolding, alteration of the glycosylation of receptors, such as the receptor for the follicle-stimulating hormone (FSH), and/or ER stress.[59,61,62] This abnormal glycosylation pattern significantly improves under a galactose-restricted diet, but there is no connection between glycosylation defects and severity of patient outcome.[59]

Another molecular mechanism underlying galactosemic complications suggested by in vivo and in vitro experiments is the oxidative stress.[41,63–67] Schulpis et al show an inverse correlation between high levels of Gal-1-P and antioxidant status, and a positive correlation between high levels of Gal-1-P and hydroxy-2-deoxyguanosine, a marker of DNA damage, in the blood of galactosemic children treated with moderate or very restricted diet.[64] In addition, experiments in rats fed with high dose of galactose showed the increase of lipid peroxidation and a decrease of antioxidant enzymes such as superoxide dismutase (SOD) and catalase in their brain.[41,65] Experiments in *Drosophila* showed an increase in oxidative stress and decrease in antioxidant defences after galactose treatment compared to control. The effects of oxidants on the survival after galactose treatment is more dramatic in *GALT*-null *Drosophila* compared to non-mutant control, suggesting a difference in how the animals respond to oxidative stress.[66] In a recent study, the authors investigate a possible link between altered glycosylation and oxidative stress. They showed that altered glycosylation can determine ER stress in human patient cells.[67] Moreover, others suggest that ER stress can downregulate the PI3K/Akt pathway in GALT deficiency directly or through altered glycosylation.[68] In fact, they showed that PI3K/Akt pathway, involving in the cell growth and metabolism regulation, is downregulated in GALT-deficient mouse fibroblasts. However, other studies are necessary to completely understand the link, if any, between oxidative stress and aberrant glycosylation. Some authors suggest that Gal-1-P accumulation can determine ER stress, in fact in human fibroblasts cell lines, Gal-1-P accumulation inhibited human inositol monophosphatase and, as a consequence, reduced IP_3 level and Ca^{2+} release from the ER.[62] However, experiments performed in a yeast model of galactosemia showed that the inhibition of inositol monophosphatase alone is not sufficient to cause ER stress and activation of the UPR.[69]

4.1 | Neurological complications

It has been hypothesized that the defect of glycosylation causes intellectual disability in galactosemic patients as well as in the CDG that are also characterized by neurological impairment.[70] Moreover, defects of glycosylation might determine the formation of abnormal myelin, which would explain the abnormality in the brain white matter in galactosemic patients.[71–74] Finally, experiments in *GALT*-deficient *Drosophila* showed modifications in the carbohydrate composition of the neuro-muscular junction synaptomatrix and consequent alterations in Wnt trans-synaptic signaling.[75] However, the role of altered glycosylation in the neurological complications is questioned because some studies have shown that white matter is also abnormal in patients without neurological complications[76] and that intellectual disability is not modified with age.[77] Moreover, at present, there is no other evidence in humans on the altered pattern found in the *GALT*-deficient *Drosophila* model.

Another mechanism suggested to be involved in the pathogenesis of neurological alteration in galactosemia is the oxidative stress. This is particularly supported by experiments in rodents showing that acute and chronic treatment with galactose in rats increases the oxidative stress in the brain.[41,42,65] Treatments with antioxidants improved the negative effects of D-galactose treatments.[78]

4.2 | Primary ovary insufficiency

Female with classic galactosemia can present primary ovary insufficiency (POI), consequent to premature depletion of follicular cells or resistance of follicles to gonadotrophin stimulation, with consequent failure of growth and maturation.[79] The mechanisms hypothesized for POI in female galactosemic patients are related to indirect or direct damage. The first could be related to the alteration of glycosylation, in particular, the hypoglycosylation of FSH and/or its receptor and probably the consequent impairment of their interaction. The analysis of galactosemic patients has showed a reduced galactosylation, and the presence of significant abnormal FSH isoforms.[80] However, this mechanism is questioned because more recent papers have contradicted these early claims.[81,82] In fact, another study did not find alteration in the FSH isoform in galactosemic females with POI compared to females with CDG or in postmenopausal women.[83] Moreover there was no significant response to the stimulation with FSH.[82]

The direct damage is related to the apoptosis of ovarian cells. Lai et al showed that rats fed with high doses of galactose showed a decreased number of oocytes ovulated and corpora lutea compared to rats fed on a normal diet. Moreover, the ovarian tissue of treated rats showed an increase in Fas and Fas-ligand (pro-apoptotic proteins) and a decrease in apoptosis inhibitors. An important role for galactitol in follicle apoptosis has been hypothesized, although there are no evidence of galactitol accumulation in human ovarian cells.[84] The pathogenetic mechanism induced by galactitol is suggested by the evidence that GALT-knockout mice present low aldose reductase levels, but galactitol accumulation is low compared to galactosemic patients. Moreover, as previously reported for the pathogenetic mechanism of cataract, galactitol determines a water influx because it

has an osmotic effect due to the difficulty to cross cell membranes, and it increases the oxidative stress through the induction of ER stress and consequent granulosa cells apoptosis.[18,53] Finally, animals fed with high doses of galactose present an increased accumulation of galactitol in the ovary, decreased oocytes maturation and inhibition of ovulation. Moreover, an aldose reductase inhibitor reduced the ovarian galactitol accumulation and the toxic effects on the oocyte.[85]

5 | ALLELE VARIANTS OF *GALT* GENE

The *GALT* gene maps to chromosome 9p13 (3 438 130-3 451 032 bp) and consists of 11 exons of about 4.3 kb. The protein encoded is a homodimer with 2 identical functional sites containing a His-Pro-His motif. Each monomer of the GALT protein consists of 379 residues.[86] More than 300 variants have been reported and listed in the ARUP *GALT* database (http://arup.utah.edu/database/galactosemia/GALT_welcome.php).[87] The most frequent severe variants in the Caucasian population are c.563A>G (p.Gln188Arg) and c.855G>T (p.Lys285Asn), with a prevalence of 70% and 54%, respectively, with respect to the total variant alleles.[88,89] The most frequent variant in African-Americans is c.404C>T (p.Ser135Leu) that has a prevalence of approximately 50% with respect to the total variant alleles.[25] Although p.Ser135Leu determines an activity of <1% in erythrocytes, it is associated with a milder phenotype, consisting of acute complications, all of which improve with a galactose-restricted diet, and the lack of long-term complications with diet treatment.[7,24] The benign outcome is probably related to a higher GALT activity present in other tissues, such as in the liver. Two large deletions, 5 and 5.5 kb-del, have been identified in Ashkenazi Jews.[90,91] The first one involves part of the promoter and exons 1 to 10,[90] while the second one is a complex variant consisting of: (1) deletion of a 3163-bp-long segment of DNA containing the 5′-end of the gene, including the promoter; (2) deletion of a 2295-bp-long segment from the 3′-end; (3) deletion of 117 bp containing portions of exon 8 and intron 8; and (4) 12 bp insertion of unknown origin downstream of the exon 8/intron 8 junction.[91]

Mutation c.940A>G (p.Asn314Asp) presents a frequency of approximately 11% in the European population and nearly 8% in the pan-ethnic population.[8] p.Asn314Asp is associated both with Duarte-1 (D1 or Los Angeles) and Duarte-2 (D2) variant alleles. In the D1, p.Asn314Asp is in cis with c.652C>T (p.Leu218Leu), a synonymous variant, while in the D2 it is in cis with 3 intronic variants (c.378-27G>C, c.507+62G>A, and c.508-24G>A), and with a deletion in the Untranslated region (UTR), c.-119-116delGTCA (5′UTR-del).[92] The increase in GALT activity in the D1 depends on p.Leu218Leu that causes overexpression of the enzyme,[93] whereas the reduction in GALT activity in the D2 is related to the instability of the GALT protein and reduced activity in human lymphoblastoid cells derived from patients homozygous for p.Asn314Asp.[94] This result could depend on the presence of other variants in cis with p.Asn314Asp, because linkage disequilibrium between p.Asn314Asp and the 3 intronic variants mentioned above has been described.[95] Thus, the reduction of GALT activity in the D2 allele seems to depend upon the presence of these 3 intronic variants or 5′UTR-del.[92] In particular, Podskarbi et al suggested that the 3 intronic substitutions may alter the mRNA.[96] in vitro experiments showed that 5′UTR-del decreases the transcription of *GALT* genes by about 55%,[97] suggesting a primary role of 5′UTR-del in GALT activity reduction in D2 patients. p.Thr23Ala, p.Gln207*, and c.564+15G A in cis with p.Asn314Asp results in null alleles, in particular, p.Asn314Asp in cis with p.Gln207* results in the formation of a truncated protein, while when it is in cis with c.564+15G>A—near the donor splice site of intron 6—it results in an RNA splicing variant.[98]

6 | GENOTYPES AND PHENOTYPES

Genotypes and phenotypes correlation is another unresolved question in galactosemia. It is hypothesized that genotype may correlate with GALT activity, which in turn may influence metabolites accumulation and phenotypes.[23] The investigators analyzed several GALT variants in yeast and identified 3 types of alleles depending on the residual GALT activity (<1%, 1%-5% and >10% of wild-type activity).[23] In particular, the alleles with an activity <1% determined in the yeast the most significant sensitivity to galactose and the most prolonged accumulation of Gal-1-P; those presenting a residual GALT activity of 1%-5% determined intermediate galactose sensitivity and transient Gal-1-P accumulation, and those presenting >10% residual GALT activity determined no sensitivity to galactose and no detectable accumulation of Gal-1-P. On the basis of the residual GALT activity, it has been also suggested the classification of the phenotype into classic or severe and variant.[7,99] In particular, the alleles resulting in a residual enzyme activity <1% are usually associated with severe galactosemia characterized by neonatal and long-term complications despite dietary treatment,[99] while alleles resulting in a residual GALT activity >1% are often associated with variant forms.[17,73,99,100] The classic example of variant form is represented by the African-American variant galactosemia (p.Ser135Leu/p.Ser135Leu), which determines a residual GALT activity <1% in erythrocytes and 10% in the liver and it is usually associated with a better outcome compared to the classic form.[7,25] Patients who were compound heterozygous for a variant that determines <1% GALT residual activity and a variant that determines a residual activity of >1% in the protein, such as p.Asp113Asn/Gln188Arg, p.Pro183Thr/Gln188Arg, p.Arg148Trp/Ser135Leu, usually present less severe neonatal symptoms compared to the classic form, and better outcome if an early diagnosis and diet is made.[101–103] Studies on D galactosemia show that a residual activity of >40% is not associated with neonatal and long-term complications. In fact, several studies have analyzed the predicted phenotype of patients with D galactosemia who are homozygous for the D allele or heterozygous for the D allele and one of the severe alleles. In particular, in homozygosity, the D1 allele does not cause galactosemia because it promotes increased GALT expression and, as a consequence, increased specific activity (>140% vs normal), while patients heterozygous for D alleles D1/D2 or homozygous for D2 show a GALT activity of 40% or 50% vs normal, respectively, and in both cases no clinical symptoms were reported.[8,89,93,96,102] However, the classification of galactosemia on the basis of residual GALT activity is

still questioned because it is based principally on yeast studies and the evidences in humans are not very clear. For example, only 3 studies have reported the outcome of patients who are heterozygous for D2 and a severe allele (genotype D/G), with contradictory results. Ficicioglu et al reported the outcome of 28 children of age 1 month 6 years, of which 17 were in a lactose-restricted diet in the first year of life, and 11 were on a regular diet since birth. They showed that none exhibited neonatal or long-term complications, in particular there were no significant differences in FSH levels and language or intelligence quotient (IQ) scores. Only in the adaptative score of neurodevelopment tests was a difference found between the 2 groups.[104] Powell et al showed that out of 75 children with D galactosemia of age 3 to 10 years and on a galactose-restricted diet in the first year of life, none presented mental disability but 5 showed speech and/or language alterations.[105] Finally, a pilot study showed that 10 patients of age 6 to 11 years with genotype D/G, on lactose-restricted diet in the first year of life, presented no mental disability but altered memory tests, auditory processing speed and one presented speech problems.[106]

7 | IN VITRO AND IN VIVO ANALYSES OF GALT VARIANT ALLELES

The studies of the effects of the allelic variants in the *GALT* gene on the residual activity of GALT enzyme include in vitro and in vivo analyses. The studies in cultured cells, in particular COS cells, are based on the analysis of residual GALT activity of a single allelic variant that is electroporated in the cells, while in the studies in yeast or bacteria 1 allelic variant derived from a galactosemic patient is typically inserted into an expression vector and expressed in genetically modified *S. cerevisiae* or *E. coli*, deficient in endogenous GALT enzyme activity.[23,107] Then, one of the following enzymatic activity assays is performed on the lysates to quantify the residual GALT activity. The fluorescent assay in erythrocytes and leukocytes of galactosemic patients is based on the detection of nicotinamide adenine dinucleotide phosphate (NADPH) fluorescence, in particular the patient samples are incubated with substrate reagent with consequent NADPH production depending on the residual GALT activity.[108,109] The radioactive assay[88,110] to measure the GALT enzyme activity in the patient blood sample using as a substrate ^{14}C-labeled Gal-1-P is used to determine the production of ^{14}C-UDP-gal that is further quantified by paper chromatography or by ion-exchange liquid chromatography. A more recent technique, liquid chromatography-tandem mass spectrometry, uses a stable isotope-labeled α-Gal-1-P as enzyme substrate. The sample is separated by reverse-phase ion-pair chromatography, and the isotopes [$^{13}C_6$]-UDP-gal and [$^{13}C_6$]-Gal-1-P produced by GALT enzyme are detected by MS/MS and quantified by using an internal standard.[111] This method is more specific and sensitive, and it measured very low enzyme activity (<5% vs normal) compared to the previous techniques. Another technique is the [1-^{13}C]Galactose Breath Testing that consists in the measure of the $^{13}CO_2$ in expired air by automated gas-isotope-ratio mass spectrophotometer after an intravenous dose of either D-[1-^{13}C]-galactose or D-[2-^{13}C]-galactose. This method allows measuring the whole-body galactose oxidation, in this way it is possible to identify the unknown allelic variants that determined different levels of GALT activity in different type of tissues.

The results of residual enzyme activities determined by the GALT allelic variants in the different systems often do not agree. In particular, the analyses performed in the yeast system often disagree with the results of experiments performed in bacterial or cellular systems. This probably depends upon the presence of alternative pathways for the metabolism of Gal-1-P in yeast or, in the case of COS cells, for the presence of residual endogenous GALT enzyme.[23,112,113]

Several GALT allelic variants has been studied in more than 1 system, such as in yeast and COS cells, others only in the erythrocyte of the patients homozygous or heterozygous for 2 different variants, others were not analyzed. In Table S1, Supporting Information, we report the residual GALT activity related to each allelic variants analyzed in at least 1 in vitro or in vivo system.

8 | IN SILICO ANALYSES OF GALT VARIANT ALLELES

An in silico approach to identify the position of the variants on the 3-dimensional structure of human GALT protein and to predict their impact on the active site, on the structural features of the protein, and on the protein stability, has been proposed in the last years and has allowed to produce data that were made available to all people interested, through a public database (GALT protein database, http://bioinformatica.isa.cnr.it/GALT/GALT2.0/).[114] The results of computational analysis on most GALT variants are in agreement with the experimental studies on the severity or benign effect of an allelic GALT variant.[16,103,115–117] This approach improves the comprehension of the structural and functional effects of the allelic variants and of the molecular mechanism by which a variant is more deleterious than another one. In particular, it was possible to argue that the benign variants have generally no or limited effect on the protein structure, altering mainly the local network of H-bonds and/or salt bridges in which the residue is involved, while the severe alleles determined an impact mainly on those residues involved in GALT intersubunit interactions or in interactions with the substrate. Several studies were also made in order to predict the effect of compound heterozygous alleles on GALT proteins, to evaluate the combined effects of the 2 variations that were not predictable by the analysis of the separated variations at protein level.[16,103,117] Sometimes, variants with no apparent effect on the static protein structure were predicted to have a deleterious effect, for example, on substrate binding and catalysis when a dynamic approach was adopted.[117] Furthermore, most allelic variants were predicted to cause protein instability with potential misfolding effects; in particular, exons 4, 7, and 9 appear to be particularly enriched in predicted destabilizing variations compared to other exons.[114]

All the computational studies made so far were based on the model of GALT human enzyme obtained by homology modeling methods starting from the structure of the *E. coli* GALT enzyme.[115] Only recently, has a crystallographic structure of the human enzyme been reported,[118] showing an overall agreement with respect to the previous model (RMSD: 0.893 Å) and with respect to the

interpretation of the structural effects of some important allelic variants, including p.Gln188Arg, but showing also some discrepancies, essentially in the conformation of some side chains of the protein. Therefore, a new project is ongoing in order to remodel all the known allelic GALT variants starting from this new structure, with the aim of providing an even more accurate interpretation of the structural/functional effects of the mutations.

9 | CONCLUSION

Without an early dietary treatment, galactosemia is a potentially life-threatening disease. The newborn screening program has reduced the death related to galactosemia, but it has failed to prevent all neonatal complications in the first few days of life.[15,16] Moreover, the early galactose-restricted diet fails to prevent severe long-term complications in patients with severe galactosemia.[89,119]

Many hypotheses for the mechanisms of neonatal and long-term complications have been reported over the decades, and which galactose metabolite as well as whether its accumulation is deleterious for the galactosemic subject is still questioned. Several studies suggest that increased levels of Gal-1-P and galactitol play a principal role in the neonatal complications, through different mechanisms depending on the type of tissue like as the inhibition of enzyme involved in the galactose metabolism,[19] or increase of osmotic stress.[46] The mechanisms proposed for long-term complications include defect of glycosylation,[58,67,75] oxidative stress[65,66,78] or epigenetic modification.[120] However, if or how Gal-1-P, galactitol or other metabolite induce these mechanisms are yet unknown, as well as if these mechanisms are independent or dependent on each other. Furthermore, some mechanisms have been questioned because of contradictory results between studies, as for the hypoglycosylation of FSH in the female galactosemic patients,[61,82] or the correlation between abnormal white matter and intellectual disabilities in galactosemic patients.[76,77]

Moreover, most of these mechanisms have been obtained in GALT-knockout mice that do not exhibit the same complications, level of galactose and its metabolites as in humans, or the experiments were performed in models of hypergalactosemia induced in rats or in cellular systems that do not present the alteration of the GALT enzyme, by exposure to high doses of galactose. Finally, the genotype-phenotype correlation of this disease is not completely clear. Several studies have analyzed the outcome of patients with alleles resulting in null enzyme or with D alleles, but no studies reported a statistical analysis of the data on genotype, biochemical and phenotype correlation. Moreover, in some case as D/G genotype contrastant results are present on the long-term phenotype and finally few studies have reported the outcome of patients with a residual enzyme activity between 1% and 40%.[101,102,121] These contradictory results need future research, in particular in humans.

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Conflict of interest

None declared.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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