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Critical Review

The Structural and Molecular Biology of Type III Galactosemia

David J. Timson
School of Biological Sciences, Queen's University Belfast, Medical Biology Centre, Belfast, UK

Summary

Type III galactosemia is a genetic disease caused by mutations in the gene encoding UDP-galactose 4-epimerase. A variety of different point mutations located throughout the gene can be responsible. The main, disease-causing effects of these mutations appear to be a reduction in the catalytic rate constant (k_{cat}) and an increase in the proteolytic sensitivity of the protein. Many of the mutations are distant from the active site of the enzyme and therefore must be assumed to affect the overall fold of the protein. Although the disease was previously classified into a severe, or generalized, form and an essentially benign, or peripheral, form this distinction has been blurred by recent work. Instead of two separate conditions it now appears that type III galactosemia is a continuum and that the symptoms will vary depending on the mutation(s) carried by the individual sufferer. This new way of looking at the disease has implications for the treatment and long term monitoring of patients.

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Keywords Leloir pathway; UDP-galactose 4-epimerase; GALE; genetic disease; galactose; short-chain dehydrogenase/reductase.

Abbreviations GALM, Galactose mutarotase; GALK1, galactokinase; GALT, galactose-1-phosphate uridyltransferase; GALE, UDP-galactose 4-epimerase; SDR, the short-chain dehydrogenase/reductase; k_{cat}, the catalytic rate constant or turnover number; K_m, the Michaelis constant.

INTRODUCTION

Galactosemias are a group of diseases caused by aberrant metabolism of the sugar galactose. In the developed world they are usually detected by routine screening of newborn babies. Since galactose, the C-4 epimer of glucose, cannot be metabolized directly by the glycolytic pathway, it is converted to glucose-6-phosphate through the Leloir pathway (Fig. 1) (*1*). This pathway consists of four enzymes: galactose mutarotase (GALM, EC 5.1.3.3), galactokinase (GALK1, EC 2.7.1.6), galactose-1-phosphate uridyltransferase (GALT, EC 2.7.7.12) and UDP-galactose 4-epimerase (GALE, EC 5.1.3.2). Mutations have been detected in these enzymes (except galactose mutarotase) which give rise to galactosemia. Although there are common features between these different deficiencies (increases in blood galactose concentrations and the development of early-onset cataracts) there are sufficient differences to distinguish three types of galactosemia. The most common form, type I galactosemia (OMIM #230400) is caused by mutations in GALT. Currently, in excess of 130 disease-associated mutations have been detected (*2*). Mutations resulting in reduced activity of GALK1 give rise to type II galactosemia (OMIM #230200). These mutations have been characterized at a biochemical and structural level (*3*, *4*). Typically, those leading to the most severe phenotypic effects result in insoluble protein (probably as a result of improper folding), whereas those leading to milder effects give rise to soluble enzyme with impaired catalytic function (*3*).

Type III galactosemia (OMIM #230350) is caused by mutations in GALE. Compared to types I and II galactosemia, only a relatively small number of patients have been characterised at the level of both genotype and phenotype. The disease was considered to exist in two forms – the severe, or generalized, form and the much milder peripheral form. Untreated sufferers of the generalized form have low (or zero) GALE activity in all tissues and typically develop cataracts within the first few months of life; these are followed by liver, kidney and brain damage (*5–7*). Current treatment for all types of galactosemia is the restriction of dietary galactose (and its precursors such as lactose). In cases of type II galactosemia, this can be quite effective. However, it is less so in the case of generalised type III galactosemia. This is likely to be in part because galactose cannot be completely eliminated from the diet in these patients as mammalian GALE is responsible not only for the interconversion of UDP-galactose and UDP-glucose but also UDP-N-acetylgalactosamine and UDP-N-acetylglucosamine. These molecules are key

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Address correspondence to: David J. Timson, School of Biological Sciences, Queen's University Belfast, Medical Biology Centre, 97 Lisburn Road, Belfast, BT9 7BL, UK. Tel: +44 (0)28 9097 5875; Fax: +44 (0)28 9097 5877. E-mail: d.timson@qub.ac.uk

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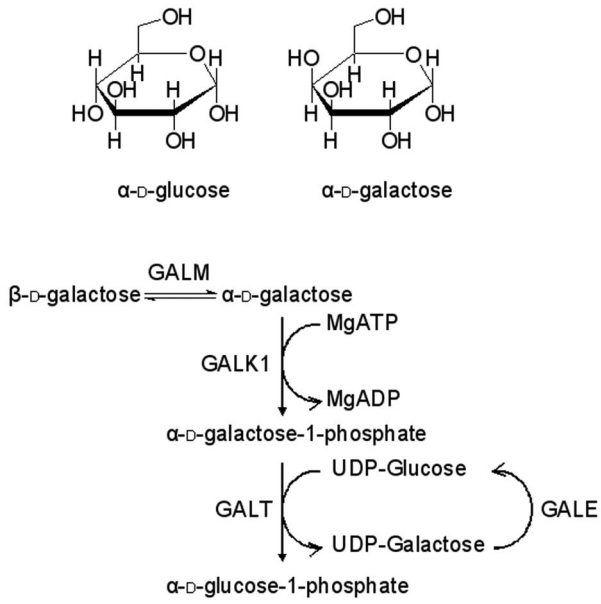

Figure 1. The Leloir pathway of galactose metabolism. Although glucose and galactose (a) differ only in their stereochemistry at position 4, a separate pathway (b) is required to convert galactose into the metabolically more useful glucose-1-phosphate. Mutations in GALT, GALK1 and GALE give rise to types I, II and III galactosemia respectively.

precursors for the synthesis of sugar moieties in glycoproteins. Indeed the disturbance of amino-sugar metabolism may be a causative factor in type III galactosemia pathology. As galactose cannot be completely eliminated from the diet, there is a build up of the toxic intermediate galactose-1-phosphate which is likely to be a second factor responsible for pathology. In contrast, in the peripheral form GALE activity is reduced in the blood, but appears normal in other tissues. The reasons for this are not known. The symptoms are much milder; indeed some patients may suffer no symptoms apart from altered levels of blood galactose and galactose-1-phosphate. In such cases no therapeutic intervention is considered necessary.

UDP-GALACTOSE 4-EPIMERASE

This enzyme, originally known as galactowaldenase, was first identified by Leloir in 1951 (*8*) and first isolated by Maxwell in 1957 (*9*). Structurally it is a member of the short-chain dehydrogenase/reductase (SDR) class of enzymes (*10*) and structures have been solved for the *Escherichia coli*, trypanosome, *Saccharomyces cerevisiae* and human enzymes (*11–17*). The enzyme is a dimer in solution with a substrate binding site located in each sub-unit (Fig. 2). Each sub-unit also contains a tightly bound NAD^+ molecule. This cofactor is required for catalytic activity since the mechanism requires the transient oxidation of the hydroxyl group on C-4 of the sugar, followed by re-reduction (*18*). Although the enzyme binds

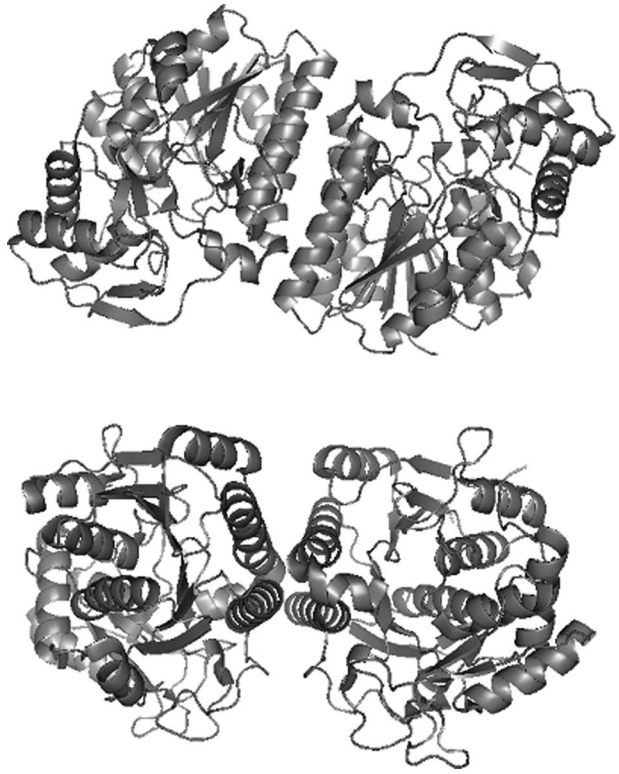

Figure 2. The structure of a human UDP-galactose 4-epimerase homodimer. The lower image is rotated approximately 90° on a horizontal axis relative to the top one. The image was generated with PyMol (DeLano Scientific LLC, San Carlos, CA, USA; http:/www.pymol.org) using PDB file 1EK6 (*13*).

tightly to the UMP moiety, it is believed that the sugar is held less tightly which permits it to move within the active site. This movement means that the re-reduction reaction is not stereospecific and thus the products are a mix of C-4 isomers (*19*, *20*). The redox reaction is facilitated by a tyrosine residue (Tyr-157 in the human enzyme) acting as an active site base (*13*, *21*). The bacterial enzyme is highly specific for UDP-galactose, whereas the human enzyme can also catalyse the interconversion of UDP-N-acetylgalactosamine and UDP-N-acetylglucosamine. This difference in specificity arises from a larger active site in the human enzyme which can accommodate the larger substrate (*14*).

MUTATIONS IN UDP-GALACTOSE 4-EPIMERASE ASSOCIATED WITH TYPE III GALACTOSEMIA

The association between a form of galactosemia and reduced UDP-galactose 4-epimerase activity was first noted by Gitzelmann (*22*). Our understanding of this link was enhanced by the determination of the sequence of the gene encoding GALE (*23*) and the subsequent characterization of disease-associated mutations. Currently, 22 such mutations

which result in amino acid changes in the protein and one which results in premature termination have been detected (*7*, *24–28*). Of these mutations, only one – V94M – is associated with the more severe, generalised form of the disease (*7*). One amino acid substitution, V180A, is commonly detected but does not appear to be associated with disease. Thus it is likely to arise either from an error in the original sequence or a polymorphism at the corresponding position in the gene (*28*, *29*). Many of these mutant proteins have been studied either as isolated proteins, in cell extracts or in model systems such as cell culture or yeast. The main results of these studies are summarized in Table 1.

The main factors in disease causation appear to be reduced catalytic efficiency (caused mainly by a reduction in the turnover number, k_{cat}) and decreased stability. This reduced stability results in a higher susceptibility of some mutant proteins to limited proteolytic digestion *in vitro* and to decreased amounts of intact protein *in vivo* (*29*, *30*). The mutation associated with the generalized form of the disease, V94M, has one of the most impaired turnover numbers (of the mutations so far characterized), but does not appear to be less stable than the wild type (as judged by limited proteolysis) (*7*, *30*, *31*). A fuller understanding of the relationship between the functional effects of the mutations and disease-causation is not possible due to two factors – the relatively small numbers of patients characterized and the fact that many galactosemic individuals are heterozygotes. For example, G90E has a turnover number which is impaired to an even greater extent than V94M and has higher proteolytic sensitivity than wild type (*30*). However, it has only been seen in a heterozygote patient who was classified as suffering from the peripheral form of the disease (*24*). However, it seems likely that if a patient were homozygous for this mutation, their symptoms would most likely be at least as severe as for V94M homozygotes.

Since GALE is a dimer, there are three possible dimers in heterozygotes (two different homodimers and a heterodimer). Although it is difficult to recreate isolated heterodimers *in vitro*, considerable insight into the function of medically relevant heterodimers has been provided by studies of different mutations co-expressed in the yeast *S. cerevisiae*. Expression of wild type human GALE complements the deletion of the yeast GALE (Gal10p) and enables growth on galactose (*29*). Co-expression of either N34S or L313M with the wild type caused reduced activity suggesting that these mutations may be (partial) dominant negatives (*29*). In contrast, co-expression of V94M with the wild type showed no dominant negative effect (*7*).

STRUCTURAL CORRELATIONS OF DISEASE-CAUSING MUTANTS

Only one disease-causing mutation, V94M, has been analysed at a structural level (*15*). In the wild type protein, Val-94 helps anchor the sugar moiety into the active site pocket. However, the structure of V94M clearly shows that this substitution causes a loop connecting an α-helix and β-strand close to the active site to become disordered (Fig. 3). This opens up the sugar binding pocket, presumably permitting non-productive binding. Since most of the non-covalent enzyme-substrate interactions involve the nucleotide moiety and not the sugar, these are relatively unaffected by this change. This explains why the major kinetic effect of this mutation is on the turnover number and not the Michaelis constant (*15*).

The other known mutations are located throughout the sequence and do not cluster into any one particular part of the structure (Fig. 4). The majority (A52V, N34S, R40C, D69E, S81R, D103G, T150M, R169W, D175N, K257R, P293L, G302D, L313M, G319E and R335H) are at least partly surface exposed. Very few are close to either the substrate (V94M and R239W) or the bound co-factor (N43S, G90E and K161N). Surface exposed mutations might be assumed to affect protein-protein interactions. However, none are located in the interface between the monomers of the GALE dimer and none of the mutant proteins tested showed any defect in dimerization (*30*). Furthermore it is possible that a failure to dimerize would not affect the enzyme's activity greatly: monomers of the *E. coli* enzyme appear to be almost as active as the dimer (*32*). There is circumstantial evidence for a multi-enzyme complex, or metabolon, of Leloir pathway enzymes in *S. cerevisiae*. In this organism the GALM and GALE enzymes are contained in a single polypeptide chain (Gal10p) (*33*). The GALT enzyme (Gal7p) localizes to discrete spots within the cytoplasm and this localization is dependent upon the presence of Gal10p and the galactokinase (Gal1p) (*34*). Although human GALT expressed in yeast does localise to spots (*34*), there is as yet no biochemical evidence for the direct physical association of the human Leloir pathway enzymes. Indeed residues in Gal10p which provide contacts between the GALE- and GALM-like domains are not well conserved in the human proteins (*17*). Based on the current evidence, it seems that the most likely explanation for the effects of these surface exposed mutations on GALE activity is that they cause perturbations of the fold which propagate to the active site or that they affect the stability of the protein.

The mutations are a diverse group of changes ranging from conservative (D69E, K257R) to charge creation (S81R, G90E, G302D and G319E), removal (R40C, D103G, K161N, D175N and R239W) and reversal (E165K) and the replacement of small residues with much bulkier ones (A25V). Thus, both in terms of the spatial distribution of the mutations and their nature there appears to be no pattern in what will result in type III galactosemia. In some cases, e.g., L183P which introduces the conformationally constrained proline into a β-sheet in the interior of the protein, it is easy to see why the mutation will result in impaired function. Indeed, in this case the mutant protein is highly susceptible to proteolysis (*30*). In other cases, the cause is less clear and only detailed structural

Table 1
Disease-associated mutations in human UDP-galactose 4-epimerase

Mutation	Type[a]	Genotype and symptoms[b]	Functional consequences[c]	References
A25V	P	Heterozygous with R169W; reduced GALE activity in blood	nd	(*27*)
N43S	P	Heterozygote with L183P; mild to moderate mental retardation at 5 years; developmental delays	Essentially wild type kinetic parameters; increased susceptibility to protease digestion *in vitro*.	(*25*, *29*, *30*)
R40C	P	Heterozygous with A25V or R169W; reduced GALE activity in blood	nd	(*27*)
D69E	P	Heterozygous with E165K or G302D; reduced GALE activity in blood	nd	(*27*)
S81R	I	Heterozygote with G319E	Reduced GALE activity in lymphoblasts	(*28*)
G90E	P	Compound heterozygote with another, uncharacterized, allele.	Cannot substitute for yeast Gal10p[d]; increased proteolysis *in vitro;* k_{cat} impaired ~800-fold compared to wild type	(*7*, *24*, *30*)
V94M	G	Homozygous; classical galactosemia symptoms at five days; deafness and moderate learning difficulties	Substitutes for yeast Gal10p, but growth is restricted; k_{cat} impaired ~30-fold compared to wild type; this is due to greater space in the active site, permitting the sugars to adopt non-productive conformations	(*6*, *7*, *15*, *30*, *31*)
D103G	P	Homozygous	Can substitute for yeast Gal10p; increased proteolysis *in vitro*; mild (~6-fold compared to wild type) impairment in k_{cat}	(*7*, *24*, *30*)
T150M	I	Homozygous	Reduced GALE activity in lymphoblasts	(*28*)
K161N	P	Heterozygote with unknown allele	Slightly reduced GALE activity in lymphoblasts	(*28*)
E165K	P	Heterozygous with D69E; reduced GALE activity in blood	nd	(*27*)
R169W	P	Heterozygous with A25V or R40C; reduced GALE activity in blood	nd	(*27*)
D175N		Heterozygote with unknown allele	Reduced GALE activity with UDP-N-acetylgalactosamine in lymphoblasts	(*28*)
L183P	P	Heterozygote with N34S; mild to moderate mental retardation at 5 years; developmental delays	Reduced protein stability when expressed in yeast or *E. coli* and *in vitro*.	(*25*, *29*, *30*)
R239W	P	Heterozygous with R335H; reduced GALE activity in blood	nd	(*27*)
K257R	P	Heterozygote	Can substitute for yeast Gal10p; mild (~6-fold compared to wild type) impairment in k_{cat}; reduced GALE activity in lymphoblasts	(*24*, *28*, *30*, *39*)
P293L	I	Heterozygote with unknown allele	Reduced GALE activity in lymphoblasts	(*28*)
G302D	P	Heterozygous with either D69E or R335H; reduced GALE activity in blood	nd	(*27*)
L313M	P	Heterozygous	Can substitute for yeast Gal10p; mild (~6-fold compared to wild type) impairment in k_{cat}	(*7*, *24*, *30*)

(*continued*)

Table 1
(*Continued*)

Mutation	Type[a]	Genotype and symptoms[b]	Functional consequences[c]	References
G319E	P	Heterozygous with either S81R or unknown allele	Can substitute for yeast Gal10p; essentially wild type kinetic parameters; reduced GALE activity in lymphoblasts	(*24*, *28*, *30*, *39*)
R335H	P	Heterozygote; detected in Korean patients heterozygous with either R239W or G302D	Slight changes in kinetic parameters compared to wild type (less than 3-fold)	(*26*, *27*, *30*)
W336X	P	Heterozygote with unknown second allele; reduced GALE activity in blood	nd	(*27*)

Notes: [a]G, generalized; I, intermediate; P, peripheral. These classifications are based on reported diagnoses. [b]Note that symptoms may be reduced by prompt treatment on galactose restricted diets. [c]No isolated enzyme studied so far has shown a change in K_m for UDP-galactose greater than threefold (*30*, *31*); GALE activity and k_{cat} values refer to activity with UDP-galactose unless otherwise stated. nd, not determined. [d]Gal10p is the *Saccharomyces cerevisiae* GALE. The protein also has GALM activity (*33*).

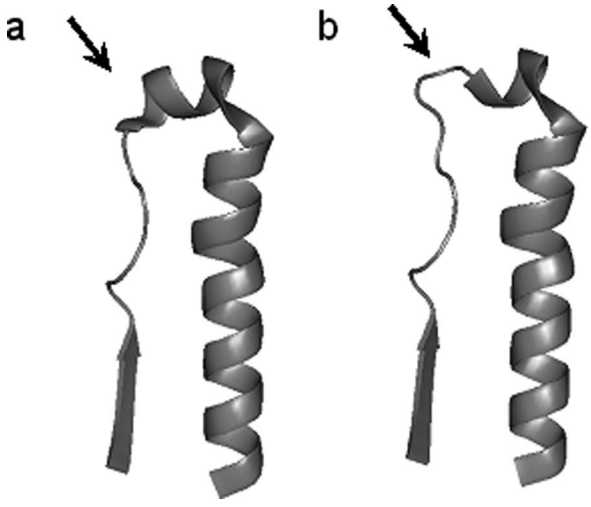

Figure 3. The region of the protein affected by the mutation V94M (residues 82 – 122). In the wild type (a), the loop joining the α-helix and β-sheet is less flexible and more ordered in comparison to the mutant (b). The position of residue 94 is indicated with an arrow. The image was generated with PyMol using PDB files 1EK6 (wild type) (*13*) and 1I3L (mutant) (*15*).

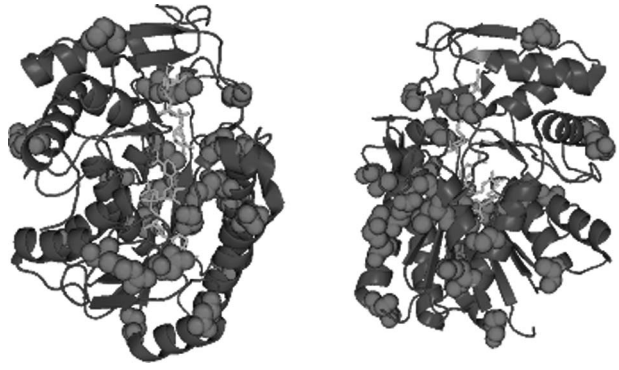

Figure 4. The structure of human GALE showing the positions of disease-associated point mutations. The backbone (blue) is shown as a cartoon and the sites of the mutations (red) as space filling models. The ligands are shown in green with UDP-glucose at the top in these diagrams. The image on the right is rotated approximately 180° on a vertical axis relative to the one on the left. Only one subunit of the homodimer is shown for simplicity. The image was generated with PyMol using PDB file 1EK6 (*13*).

or modelling studies are likely to reveal the links between sequence changes and reduction in enzymic activity.

THERAPIES AND TREATMENTS

The usual therapy for all types of galactosemia is to restrict the dietary intake of galactose and its precursor lactose. However, this is an unsatisfactory treatment in the case of type III galactosemia as small amounts of galactose are required for the biosynthesis of UDP-galactose. An ideal alternative to this treatment would be some form of gene therapy which enabled the partial restoration of GALE activity. This could take the form of direct replacement of the *GALE* gene or the re-engineering of alternative pathways of galactose metabolism as has been suggested for GALT-deficiency (*35*). Even if the treatment, in effect, caused the conversion of symptoms from the generalized form to the peripheral, it would be a great improvement in the quality and quantity of life for the patients. However, the relatively small number of patients and recent problems with gene therapy technologies (*36*) mean that

this solution is unlikely to be available in the near future. In the case of type I galactosemia, where the build-up of galactose-1-phosphate is believed to be a major factor in parthenogenesis, it has been suggested that inhibition of GALK1 might be a viable therapeutic strategy (*37*). Although this would mimic the effects of type II galactosemia, the symptoms of this are quite mild and overlap with those of type I. A similar strategy would, presumably, reduce galactose-1-phosphate levels in type III galactosemia. However, it would not address the failure to produce UDP-galactose and UDP-N-acetylgalactosamine in these patients. This problem would have to be addressed in any potential therapy for type III galactosemia.

FUTURE PERSPECTIVES

The most puzzling outstanding question in the study of type III galactosemia is the origin of the difference between the generalized and peripheral forms of the disease. It is not understood why, in the peripheral form of the disease, GALE activity is reduced in the blood yet appears normal in other tissues. One explanation is that there is a difference in the level of expression of the *GALE* gene in blood compared to other tissues (*24*). No mechanism has been proposed to explain this and it seems unlikely that such a wide range of different mutations, located throughout the gene would all reduce the level of expression. Furthermore replication of many of the mutations in a yeast expression system – i.e., using non-human promoters in a non-human expression system – results in defects in galactose metabolism and reduced GALE protein stability (*7*, *29*, *31*). Therefore, it seems likely that the main cause(s) of type III galactosemia is to be found at the protein level.

Although the disease was originally classified into two forms, generalized and peripheral, it has been clear for some time that the distinction between the two forms is not clear cut. Some of the patients diagnosed with the peripheral form of the disease have shown symptoms beyond altered blood chemistry (*25*, *29*). Generally these symptoms appear later than in the generalized form of the disease. However, the existence of such cases calls into question the practice of permitting those diagnosed with the peripheral form to continue on non-lactose restricted diets. It certainly underlines the need for continued surveillance of these individuals throughout childhood, and possibly beyond. A recent paper from Fridovich-Keil's group has demonstrated convincingly that type III galactosemia is not a binary condition. They showed that there was a range (15–64% of controls) of GALE activities in lymphoblasts derived from patients with the peripheral form of the disease (*28*). Lymphoblasts were used as a model as they had previously been shown to have GALE activities similar to non-peripheral tissues (*38*). Furthermore, these lymphoblasts showed statistically significantly different levels of galactose utilization in a 24-h period and increased (but different) levels of galactose-1-phosphate accumulation (*28*). The wide variation in both these results suggests that the patient's symptoms will be largely dependent upon his/her genotype and that a simple classification into generalized and peripheral is no longer appropriate. Indeed, it would be surprising if the wide range of mutations which can cause impaired GALE activity all resulted in exactly the same type and level of impairment. It will, therefore, be important that the full genotype of all new sufferers is ascertained in order to inform their treatment.

The availability of the gene sequence and the protein structure for GALE has enabled significant advances in our understanding of type III galactosemia. To extend this understanding we will need more structural data on mutants which are associated with different symptoms. We will also need more biochemical studies on the mutant enzymes in order to enhance our understanding of genotype-phenotype correlations. These biochemical studies should include experiments to determine how the disease-causing mutations affect the system of enzymes that catalyse the Leloir pathway as well as isolated GALE. These studies will be most informative when considered alongside *in vivo* models such as the yeast and lymphoblast systems. In this way an integrated picture of the biochemical and physiological causes of the disease can be developed.

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