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## COMMUNICATION

## Disturbed cofactor binding by a novel mutation in UDP-galactose 4'-epimerase results in a type III galactosemia phenotype at birth.

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Received 00th January 20xx,  
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

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UDP-galactose 4'-epimerase (GALE) is an essential enzyme in galactose metabolism and its dysfunction results in type III galactosemia. Therein we report a patient born with abnormal blood galactose levels and reduced GALE activity who was shown to be heterozygous for a c.266C>T missense mutation in the GALE gene, predicted to result in the amino acid exchange p.A89V. Over a period of months, the patient's blood galactose, galactose 1-phosphate and GALE activity levels reverted to normal, encouraging us to investigate this mutation: Structurally Ala89 is a highly conserved residue located close to the binding site of the cofactor, NAD<sup>+</sup>. Consequentially molecular modelling predicted that this mutation results in steric clashes between the cofactor and valine side chain, and bioinformatic predictions suggested that p.A89V is likely to be less stable than the wild-type. Biochemical studies on the recombinant p.A89V enzyme demonstrated lower activity than the wild type ( $K_m$  increased by approximately 30-fold;  $k_{cat}$  reduced approximately 180-fold), and additionally changes in stability and altered NAD<sup>+</sup> binding were observed. Thus, a picture emerges in which this mutation leads to reduced stability, disturbed cofactor binding and subsequently reduced activity. Overall this study suggests that bioinformatic predictions are useful in assessing the effects of newly discovered mutations on enzyme function, but care should be taken in extending predictions to the clinical phenotype especially in cases of heterozygosity. It also raises interesting questions about a

dominant negative effect of some GALE missense alleles and potential compensatory mechanisms occurring in people born with clinical chemistry measurements suggesting a diagnosis of galactosemia.

**Keywords:** GALE, UDP-glucose 4'epimerase, protein stability, NAD<sup>+</sup> binding, bioinformatics prediction, genotype-phenotype correlation

### Introduction

Type III galactosemia (OMIM#230350) is an inherited metabolic disease caused by a deficiency in the activity of UDP-galactose 4'-epimerase (GALE, EC 5.1.3.2)<sup>1-3</sup>. This enzyme catalyses the inter-conversion of UDP-galactose and UDP-glucose, a reaction that is part of the Leloir pathway of galactose catabolism<sup>4,5</sup>. Additionally GALE has a second role in maintaining the pools of UDP-galactose and UDP-glucose (along with the related metabolites, UDP-N-acetylgalactosamine and UDP-N-acetylglucosamine), which are precursors in the synthesis of the sugar moieties of glycoproteins and glycolipids. The enzyme is a member of the short-chain dehydrogenase (SDR) family utilising a bound NAD<sup>+</sup> cofactor and in regards to type III galactosemia the majority of mutations are missense, present throughout its structure<sup>3</sup>. There is a wide spectrum of symptoms associated with the disease ranging from increased concentrations of galactose metabolites without clinical effects in the mildest forms to irreversible organ damage leading to physical and cognitive disability in the most severe forms<sup>1-6-8</sup>. Currently, the only treatment is the removal of galactose (and its precursors such as lactose) from the diet. This treatment is unsatisfactory since, for those with the most severe forms of the disease, it only delays and reduces the extent of organ damage but cannot prevent or reverse it<sup>7</sup>.

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Electronic Supplementary Information (ESI) available. See DOI: 10.1039/x0xx00000x

Biochemically, disease-associated mutations tend to reduce the activity of the enzyme<sup>9-12</sup>. There is an approximate correlation between the extent of impairment in the turnover number ( $k_{cat}$ ) and the severity of disease<sup>10,12</sup>. The majority of mutations alter residues away from the active site and the loss of activity is most likely caused by altered protein stability rather than direct effects on the active site residues<sup>9,10,12-17</sup>. Interestingly both increased and decreased stability can be associated with reduced activity and disease<sup>17</sup>. GALE is a homodimer with one molecule of NAD<sup>+</sup> bound per subunit<sup>18</sup>. This NAD<sup>+</sup> is critical for the enzyme's activity and, in some of the disease-associated variants, one effect of reduced affinity for NAD<sup>+</sup> is altered stability<sup>13,17,19,20</sup>.

To date, relatively few disease-associated mutations have been reported and GALE deficiency is considered to be rare. The incidence is strongly population dependent where it is estimated to be 1:6,700 in African Americans but approximately 1:64,800 in Americans of European descent<sup>21</sup>. However, the benign (asymptomatic) nature of the milder forms of the disease may mean that there are a significant number of undetected cases in the general population. Here we report the identification of a previously unreported mutation in *GALE*, which was associated with type III galactosemia at birth along with the characterisation of this variant protein. We also demonstrate the strengths of using bioinformatics methods to predict the biochemical effects of this mutation.

## Materials and Methods

### *Molecular genetics*

*GALE* mutation analysis was carried out by PCR amplification of the coding exons and adjacent intron regions (RefSeq NM\_000403.3) followed by bidirectional Sanger sequencing using standard methods. The genetic diagnostic analyses were performed with full written informed consent in compliance with the relevant laws and institutional guidelines.

### *Bioinformatics and molecular modelling*

The crystal structures of human GALE with UDP-glucose and UDP-N-acetylglucosamine bound (PDB: 1EK5 and 1HZJ<sup>18,22</sup>) were energy minimised and solvated using YASARA<sup>23</sup>. PyMol ([www.pymol.com](http://www.pymol.com)) was used to alter residue 89 to valine in both structures and the resulting variant structures re-minimised using YASARA. The minimised models are presented as supplementary data to this paper and were used in subsequent analyses. Accessible surface areas were calculated using GETAREA (<http://curie.utmb.edu/getarea.html>)<sup>24</sup> and root mean squared deviations (RMSD) using the Align function in PyMol. Multiple sequence alignments were calculated using ClustalW<sup>25</sup> using reviewed GALE sequences in UniProt ([www.uniprot.com](http://www.uniprot.com)). Conservation scores were obtained using the Scorecons server ([http://www.ebi.ac.uk/thornton-srv/databases/cgi-bin/valdar/scorecons\\_server.pl](http://www.ebi.ac.uk/thornton-srv/databases/cgi-bin/valdar/scorecons_server.pl))<sup>26</sup> and SIFT tolerance scores from <http://sift.jcvi.org/><sup>27</sup>. Instability and aggregation tendencies were estimated using SNP effect 4.0 (<http://snpeffect.switchlab.org/>)<sup>28</sup> and I-Mutant 3.0 (<http://gpcr2.biocomp.unibo.it/cgi/predictors/I-Mutant3.0/I-Mutant3.0.cgi>) at 37 °C and pH 8.8 in order to match the conditions used in *in vitro* enzyme assays<sup>29,30</sup>.

### *Expression, purification and kinetic analysis of wild-type and variant human GALE*

Wild-type human GALE was expressed in, and purified from, *Escherichia coli* HMS174(DE3) as previously described<sup>10</sup>. The coding sequence was altered so that codon 89 encoded valine instead of alanine by site-directed mutagenesis using the QuikChange method<sup>31</sup>. The variant protein was expressed and purified using the same protocol as the wild-type. Enzyme activity was measured in 50 mM Hepes-OH, pH 8.8 at 37 °C by coupling the production of UDP-glucose to its oxidation in an NAD<sup>+</sup>-requiring reaction catalysed by UDP-glucose dehydrogenase<sup>10,32</sup>. Recombinant human UDP-glucose dehydrogenase was produced as previously described<sup>16</sup>. Protein concentrations were estimated by the method of Bradford using BSA as a standard<sup>33</sup>.

### *Analytical methods*

Limited proteolysis was carried out using trypsin (10–900 nM supplemented with 2 mM calcium chloride) and GALE (16  $\mu$ M) in a total volume of 10  $\mu$ l. UDP-galactose (2 mM) was added as required. Reaction mixes were pre-incubated at 37 °C for 5 min prior to addition of the protease. They were then incubated for a further 30 min at 37 °C before being stopped by the addition of an equal volume of SDS-PAGE loading buffer (125 mM Tris-HCl, pH 6.8, 4 % (w/v) SDS, 20 % (v/v) glycerol, 1 % (w/v) dithiothreitol, 0.002 % (w/v) bromophenol blue) and heating at 95 °C for approximately 3 min. Reactions were analysed by 10% SDS-PAGE.

Crosslinking of GALE (10  $\mu$ M) with *bis*(sulphosuccinimidyl) suberate (BS<sup>3</sup>, 0.1–800  $\mu$ M) was carried out at 37 °C in a reaction volume of 10  $\mu$ l. Reaction mixes were pre-incubated for 5 min prior to the addition of crosslinker. Reactions were allowed to proceed for another 30 min at 37 °C before being stopped by addition of an equal volume of SDS-PAGE loading buffer and heating at 95 °C for approximately 3 min. Reactions were analysed by 10% SDS-PAGE.

Melting temperatures ( $T_m$ ) of GALE (5  $\mu$ M) were estimated (in triplicate) by differential scanning fluorimetry (DSF) using Sypro Orange (Sigma) as previously described<sup>17,34</sup>. Fluorescence spectra of GALE (200  $\mu$ l of 20  $\mu$ M) were collected in triplicate using a Spectra Max Gemini X UV platereader using an excitation wavelength of 280 nm.

## Results and Discussion

### *Clinical chemistry and molecular genetics*

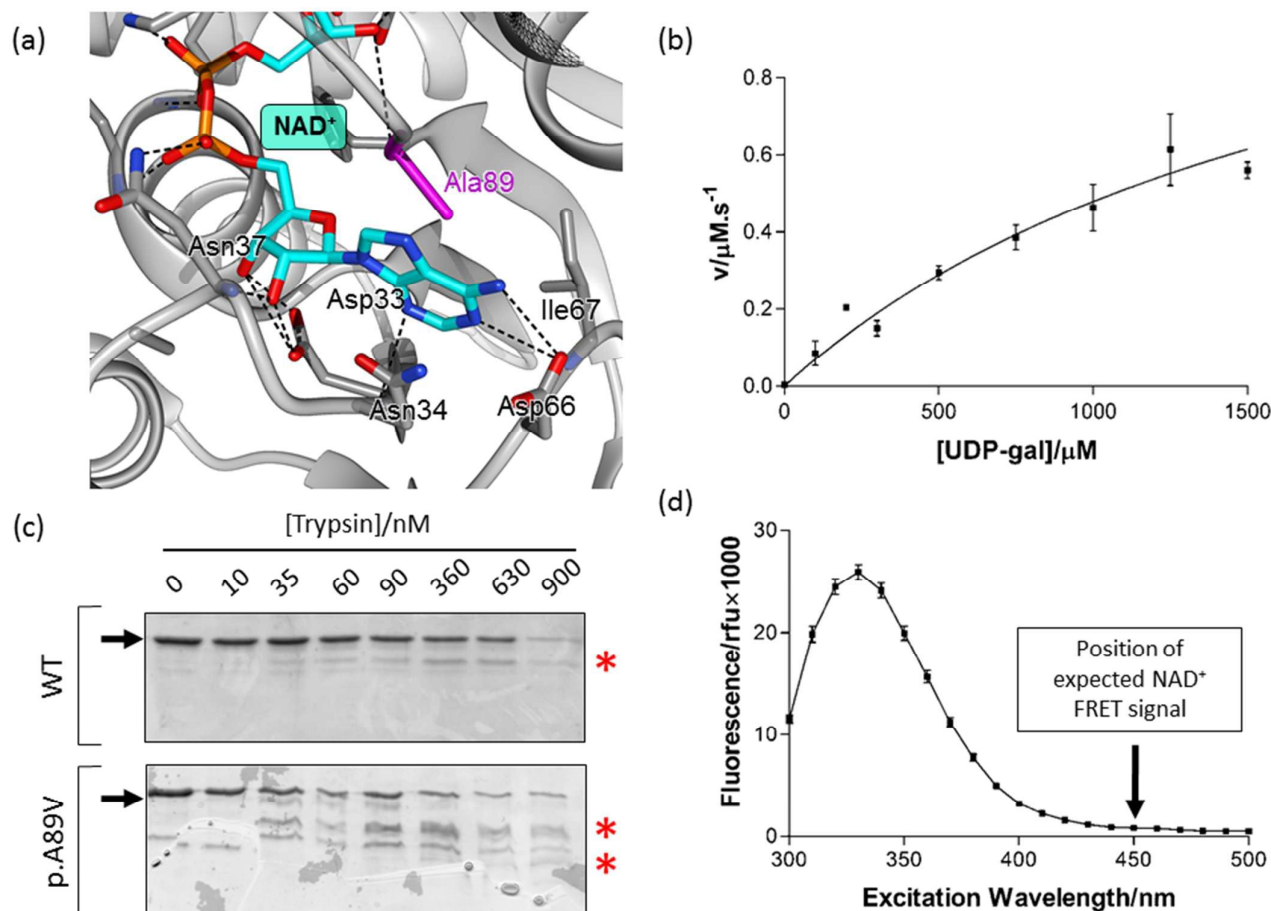
The female patient was tested for blood galactose in the neonatal period. An initial reading of 34.2 mg/dl was recorded (normal values <20 mg/dl). Eight days later this had dropped slightly to 31.2 mg/dl. At six weeks, galactose 1-phosphate levels were 6.3 mg/100 ml erythrocytes (normal < 1.5 mg/100 ml) and GALE activity was 0.41  $\mu$ mol/h.ml erythrocytes (normal 2.72–7.91  $\mu$ mol/h.ml erythrocytes). Galactokinase and galactose 1-phosphate uridylyltransferase activity were both in the normal ranges. By eight months, galactose levels were 1.3 mg/dl and galactose 1-phosphate levels were 0.98 mg/dl (both within the normal range). At 21 months, galactose 1-phosphate had dropped further to

0.75 mg/dl. The patient is developing normally without any treatment or dietary intervention.

DNA sequencing revealed a heterozygous *GALE* variant c.266C>T (rs201437329) which is predicted to cause replacement of the amino acid alanine at residue 89 with valine, denoted p.Ala89Val. Ala-89 is located in the NAD<sup>+</sup> binding site (Figure 1a). The mutation has been registered with allele frequencies of 0.4 % (29/6604) and 0.01 % (8/65860), respectively, in Finnish and non-Finnish European individuals (ExAC database accessed 23<sup>rd</sup> Sept 2015, <http://exac.broadinstitute.org>) but to our knowledge has not yet been reported in a patient with *GALE* deficiency. No other alteration of potential functional relevance in the gene was identified.

### *Bioinformatics and molecular modelling*

Multiple sequence alignment showed that Ala89 is highly conserved across *GALE* orthologues (Supplementary Figure S1). Indeed the conservation score<sup>26</sup> was 0.99 and the SIFT tolerance score<sup>27</sup> was 0.0 indicating a highly conserved residue in a site that is intolerant to this relatively conservative change. Molecular modelling predicted no significant change in the overall structures (RMSD of 0.297 Å and 0.345 Å for the UDP-glucose and UDP-*N*-acetylglucosamine bound structures respectively) and the accessible surface area of residue 89 was also similar (14.0–15.6 %) for both WT and p.A89V bound to both product molecules (Supplementary Table S1). Ala89 is located in the NAD<sup>+</sup> cofactor-binding site, adjacent to the adenine moiety and the alteration of this residue to valine introduces a larger side-chain that results in a steric clash with the adenine moiety (Figure 1a; Supplementary Figure S2). This indicates a potential to disrupt cofactor binding. Furthermore predictions with SNP effect 4.0 and I-Mutant 3.0 suggested that p.A89V is less stable than the wild-type, with an increased tendency to aggregate (Supplementary Table S2). Collectively when compared to similar analyses carried out on variants for which the degree of enzymatic impairment is known<sup>35</sup>, these results suggest that p.A89V is likely to be severely impaired compared to wild-type.



**Figure 1: Biochemical analysis of GALE p.A89V.** (a) Ala89 is located in the NAD<sup>+</sup> binding site of the protein, close to the adenine moiety. grey ribbon, backbone; grey sticks, key residues forming hydrogen bonds with NAD<sup>+</sup>; purple, Ala89; cyan, NAD<sup>+</sup>; black dashed lines, predicted hydrogen bonds. The image was generated in Chimera using PDB file 1EK5. (b) p.A89V (7  $\mu\text{M}$ ) has impaired kinetics compared to wild-type. Each point represents the mean of three determinations of the rate and the error bars the standard deviations of these means. The line represents a non-linear fit to the Michaelis-Menten equation. (c) p.A89V (16  $\mu\text{M}$ ) has reduced

1b) with a Michaelis constant ( $K_m$ ) increased almost 30-fold to  $1900 \pm 960 \mu\text{M}$  (compared to  $69 \mu\text{M}$  for the wild-type<sup>10</sup> and a turnover number ( $k_{\text{cat}}$ ) reduced 180-fold to  $0.2 \text{ s}^{-1}$ . This is less than the value measured for the p.V94M variant ( $1.1 \text{ s}^{-1}$ ; cf the wild type value of 36

stability towards proteolytic digestion compared to the wild type. Results were analysed by 10% SDS-PAGE and the sizes of molecular mass markers are shown to the left of the gel in kDa. Red asterisks indicate degradation fragments for WT and p.A89V GALE. Arrows indicate full-length GALE protein. (d) Detection of NAD<sup>+</sup> binding in p.A89V (20  $\mu\text{M}$ ) by FRET. The lack of signal at 450 nm indicates there is no NAD<sup>+</sup> cofactor bound. Each point represents the mean of three determinations and the error bar the standard deviation of those means.

#### Biochemical analysis of recombinant p.A89V

To gain insight into the biochemically effects of this mutation p.A89V was produced recombinantly and purified. The mutation was substantially kinetically impaired compared to the wild-type enzyme (Figure  $\text{s}^{-1}$ ) which is associated with severe forms of the disease<sup>10</sup>.

Like the wild-type, p.A89V formed homodimers as judged by protein-protein crosslinking (data not shown), although there was a significant change in the protein's thermal stability ( $T_m$  of  $48.8 \pm 0.7 \text{ }^\circ\text{C}$  compared to  $52.8 \pm 0.3 \text{ }^\circ\text{C}$  for the wild-type under the same



conditions;  $p = 0.0008$ , unpaired t-test)<sup>17</sup>. p.A89V was also more susceptible to proteolysis by trypsin (Figure 1c) where the variant was degraded into more fragments and showed noticeable degradation at a lower protease concentrations. Both the wild-type and p.A89V were protected from this proteolysis in the presence of UDP-galactose (Supplementary Figure S3). Moreover in the wild-type protein, the bound NAD<sup>+</sup> can be detected by FRET: when GALE is excited at 280 nm there is a large fluorescence emission peak at ~340 nm (resulting from tryptophan and other aromatic residues) and a smaller peak at ~450 nm (resulting from the bound NAD<sup>+</sup>)<sup>17</sup>. In p.A89V, the first peak was observed, but not the second (Figure 1d). Therefore recombinant p.A89V, in contrast to the wild-type, likely does not contain appreciable amounts of NAD<sup>+</sup>. This suggests that the variant has reduced affinity for the cofactor, which is likely to be the main cause of the reduced stability towards proteases and the loss of activity compared to the wild-type. Therefore any activity that could be observed most likely results from the high concentrations of NAD<sup>+</sup> (4 mM), which are necessarily present in the coupled enzyme assay.

## Conclusions

Taken together, these results suggest that the ultimate cause of the initial GALE deficiency observed here results from the additional bulk of the valine side chain compared to that of alanine. This likely reduces the affinity for NAD<sup>+</sup> substantially, and consequently the stability and activity of the protein. These biochemical results largely validate the predictive framework based on bioinformatic analyses that were previously proposed to assess newly discovered mutations<sup>35</sup>. The patient initially presented with clinical chemistry measurements consistent with a diagnosis of type III galactosemia, but she remained asymptomatic and her metabolite and enzyme activity measurements are now in the normal range.

Prolonged biochemical abnormalities and marked reduction in GALE activity are unusual in heterozygotes of GALE mutations. As such no relevant second alteration (on the other allele) was identified although a mutation in an intron, promoter or a large structural aberration involving parts of the gene cannot be excluded. Our analysis of the p.A89V mutation was not

successful in determining the cause of the patient's apparent temporary galactosemic phenotype but it is possible that the raised galactose and galactose 1-phosphate levels in the period immediately after birth in this patient could be due to a dominant negative effect. The effects appear to have been more pronounced in the neonatal period and it is likely that the patient's milk intake decreased over the period in question that may provide a partial explanation although there may also be other, as yet unidentified, compensatory mechanisms at the cellular and molecular level. Nonetheless, in support of our hypothesis, dominant negative effects have been reported for the GALE mutations p.N34S and p.L183P when heterozygously expressed in a yeast model with the wild-type. These mutations resulted in 37% and 24% respectively of the activity of strains homozygous for wild-type GALE in contrast to the expected 50%<sup>13</sup>. It also interesting to note that p.N34S affects a residue involved in NAD<sup>+</sup> binding and p.L183P likely disturbs the overall structure of this region of the protein<sup>35</sup> suggesting a link due to cofactor binding. Gaining insight into the structural and biochemical basis of this dominant negativity should be a priority for future research, especially as the majority of individuals with type III galactosemia are compound heterozygous<sup>3</sup>. Such studies would give deeper insight into the role allelic heterogeneity plays as a whole in many metabolic disorders.

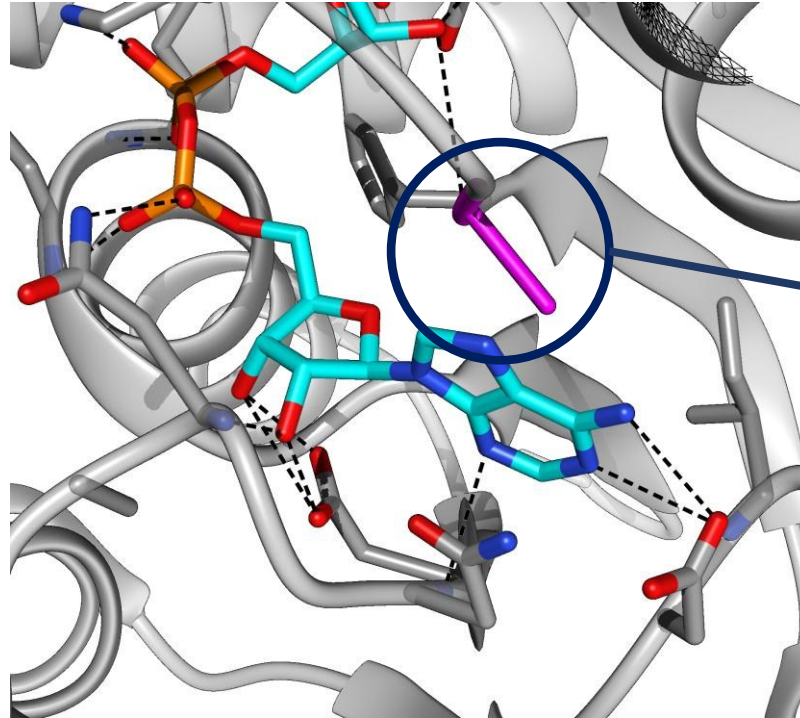
## Acknowledgements

We thank Prof Aaron Maule (Institute for Global Food Security, Queen's University Belfast) for access to a qPCR machine used in DSF assays, and Dr Ursula Albrecht (University Children's Hospital Innsbruck, Austria) who cared for the patient and arranged the diagnostic work-up. TJM was funded by a Department of Employment and Learning, Northern Ireland (DELNI, UK) PhD studentship.

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GALE: Ala-89 → Val-89

- Reduced NAD<sup>+</sup> binding
- Less stable protein
- Lower enzymatic activity
- Type III galactosemia