European Journal of Human Genetics (2016) 24, 976–984
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www.nature.com/ejhg

ARTICLE

Classical galactosaemia: novel insights in IgG *N*-glycosylation and *N*-glycan biosynthesis

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Classical galactosaemia (OMIM #230400), a rare disorder of carbohydrate metabolism, is caused by a deficient activity of galactose-1-phosphate uridyltransferase (EC 2.7.7.12). The pathophysiology of the long-term complications, mainly cognitive, neurological and female fertility problems remains poorly understood. The lack of validated biomarkers to determine prognosis, monitor disease progression and responses to new therapies, pose a huge challenge. We report the detailed analysis of an automated robotic hydrophilic interaction ultra-performance liquid chromatography *N*-glycan analytical method of high glycan peak resolution applied to serum IgG. This has revealed specific *N*-glycan processing defects observed in 40 adult galactosaemia patients (adults and adolescents), in comparison with 81 matched healthy controls. We have identified a significant increase in core fucosylated neutral glycans ($P<0.0001$) and a significant decrease in core fucosylated ($P<0.001$), non-fucosylated ($P<0.0001$) bisected glycans and, of specific note, decreased *N*-linked mannose-5 glycans ($P<0.0001$), in galactosaemia patients. We also report the abnormal expression of a number of related relevant *N*-glycan biosynthesis genes in peripheral blood mononuclear cells from 32 adult galactosaemia patients. We have noted significant dysregulation of two key *N*-glycan biosynthesis genes: *ALG9* upregulated ($P<0.001$) and *MGAT1* downregulated ($P<0.01$) in galactosaemia patients, which may contribute to its ongoing pathophysiology. Our data suggest that the use of IgG *N*-glycosylation analysis with matched *N*-glycan biosynthesis gene profiles may provide useful biomarkers for monitoring response to therapy and interventions. They also indicate potential gene modifying steps in this *N*-glycan biosynthesis pathway, of relevance to galactosaemia and related *N*-glycan biosynthesis disorders.
European Journal of Human Genetics (2016) **24,** 976–984; doi:10.1038/ejhg.2015.254; published online 6 January 2016

INTRODUCTION

Classical galactosaemia (OMIM #230400), a rare disorder of carbohydrate metabolism, is caused by deficiency of galactose-1-phosphate uridyltransferase (GALT) (EC 2.7.7.12) because of mutation of the *GALT* gene (NG_009029.1). If left untreated, the disease is life threatening in neonates. Life-long galactose restricted diet is the only treatment currently available and applied to all galactosaemia patients. Although this is life saving in the neonate, long-term complications including cognitive impairment, neurological and speech abnormalities, and fertility problems in female patients, persist in treated patients despite early diagnosis, initiation of treatment and shared genotypes. The cause of the complications remains poorly understood.[1–3]

Reduced GALT activity results in decreased UDP-galactose and the toxic build-up of intermediates of the galactose metabolism pathway. GALT maintains the balance between UDP-glucose (glc), UDP-galactose (gal), *N*-acetylgalactosamine (GalNAc) and *N*-acetylglucosamine (GlcNAc). These four UDP-hexoses are rate limiting for the biosynthesis of glycoproteins and proteoglycans, which form the basis of the extracellular synaptomatrix of the synaptic and the perisynaptic space.[4–6]

In a *Drosophila* disease model of galactosaemia, loss of GALT has been shown to impair movement coordination. Abnormalities were identified at the neuromuscular junction, as well as depletion of galactosyl/*N*-acetylgalactosamine and fucosylated moieties, which are suggested to result from limited UDP-sugar bioavailability.[7] Defective glycosylation is known to impair neurodevelopment and neurological function.[8]

The use of glycan profiling is now increasingly used as a prognostic and diagnostic biomarker in a number of diseases, including cancer, diabetes and rheumatoid arthritis.[9–11] High-throughput technology is hereby used.[12–14]

IgG is one of the most abundant glycoproteins in human plasma. The biological activity of IgG is regulated by the *N*-glycans attached to the highly variable Fc domain.[15] Changes in core fucose and sialic acid content of IgG can lead to very substantial functional changes as seen in the antibody-dependent cellular cytotoxicity activity or conversely, may confer anti-inflammatory properties.[16]

It is established that before and after initiation of treatment, galactosaemia patients exhibit defects in both assembly and processing of *N*-glycans.[17–21] These defects resemble those observed in a number of congenital disorders of glycosylation (CDG) type I (*N*-glycan

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Received 11 August 2015; revised 20 October 2015; accepted 27 October 2015; published online 6 January 2016

assembly defects) and II (*N*-glycan processing defects).[22] A combination of increased levels of galactose-1-phosphate (gal-1-p) and decreased UDP-hexose substrates causes ER stress and in turn disrupts glycosylation,[23–27] causing systemic genomic dysregulation.[19,21]

We have proposed that early-stage developmental and also later ongoing disruption of glycosylation and related gene expression pathways may have a role in modifying the observed clinical outcome in classical galactosaemia. Abnormal glycosylation during prenatal galactose intoxication is proposed to affect long-term neurological development. It is not clear if ongoing glycosylation abnormalities further modify the observed outcomes.[21,28]

In our previous studies of circulating IgG *N*-glycans, we demonstrated correction of the gross assembly defects in young galactosaemia patients following treatment with galactose restriction.[28] We also demonstrated the presence of IgG *N*-glycan processing defects in adults and children with galactosaemia maintained on a galactose restricted diet using IgG galactose incorporation ratios.[19,20,28,29] We have also demonstrated modification of the defective glycosylation pathway with moderate galactose relaxation in a number of adults and children with galactosaemia, suggesting the presence of substantial variation in accessory glycosylation pathways in some patients, which may be modifiable with substrate (galactose exposure).[20,28,29]

Our gene expression and protein findings suggest that there may also be a partial *N*-glycan assembly defect in treated adult patients that is at risk of being exacerbated during galactose intoxication.

The measurement of urinary galactitol and red blood cell gal-1-p does not reliably detect minor deviations in the diet or differentiate between levels of metabolic control in treated patients (beyond monitoring the initial effect of galactose withdrawal in intoxicated neonates.[30] However, we have now used, IgG *N*-glycan profiling to analyse galactose incorporation ratios in children and adults, which have shown consistent individual responses to diet liberalisation[1,2,20,30,31] using our recently reported improved automated hydrophilic interaction ultra-performance liquid chromatography (HILIC-UPLC) method of glycan analysis.[32,33] In the present work, we report the improved glycan peak (GP) resolution utilising this method and a comprehensive *N*-glycan analysis of total undigested serum IgG used to study processing defects in 33 adult and 7 adolescent classical galactosaemia individuals and 81 matched healthy controls. Furthermore, we report the abnormal expression of a few relevant *N*-glycan biosynthesis genes in peripheral blood mononuclear cells (PBMCs) from affected adult galactosaemia individuals.

MATERIALS AND METHODS

Study subjects and characterisation

The study of IgG analysis included 32 Irish classical galactosaemia patients and 8 Dutch patients. The characteristics of the study subjects are detailed in Table 1. The Full Scale Intelligence Quotient (FSIQ) range for the Irish and Dutch patients at last testing is detailed in Supplementary Table 1. The controls consisted of 81 healthy adults, 25 Irish adults obtained from a healthy population Health Insurance screening panel and 56 Scottish healthy controls, from an Orkney Islands, Scotland healthy population epidemiological study.

In addition, RNA from PBMCs was extracted from 32 unrelated classical adult galactosaemia patients. The characteristics of the study subjects are described in Supplementary Table 2. RNA was also banked from 10 matched healthy adult controls (Table 2).

All galactosaemia study patients were maintained on a dietary galactose intake of <500 mg gal/day. Ethical approval for this study was obtained from the ethics committee of the Children's University Hospital, Dublin, Ireland and the ethics committee of Maastricht University Hospital, Maastricht, The Netherlands. The control samples were obtained by voluntary donation, with informed consent given at the time of collection.

IgG *N*-glycan analysis

The isolation of IgG from whole serum, removal of *N*-linked glycans from IgG, 2-aminobenzamide, labelling of *N*-glycans and HILIC-UPLC methods are described in detail in Stöckmann *et al*.[32]

Table 1 IgG study patient clinical characteristics

Group	*Healthy control*	*Galactosaemia*	
Ethnicity	Irish and Scottish	Irish	Dutch
Patients	81	32	8
Age	27 (Irish: 18–36), 32 (Scottish: 20–44)	25 (13–36)	20 (14–26)
Gender	NA	14F, 18 M	5F, 3 M
Genotype (nucleotide annotation)	NA	28: (c.563A>G/c.563A>G) 2: (c.563A>G/c.997C>T) 2: (c.563A>G /c.855G>T)	8: (c.563A>G/c.563A>G)
Genotype (protein variant)	NA	28: (p.Q188R/p.Q188R) 2: (p.Q188R/p.R333W) 2: (p.Q188R/p.K285N)	8: (p.Q188R/p.Q188R)
FSIQ	NA	87 (47–126)	77 (56–97)

Table 2 Gene array patient clinical characteristics

Group	*Healthy control*	*Galactosaemia*	
Ethnicity	Irish	Irish	Dutch
Patients	10	26	6
Age	30 (19–41)	33 (17–49)	21 (18–23)
Gender	6F, 4M	16F, 10M	4F, 2M
Genotype (nucleotide annotation)	NA	21: (c.563A>G/c.563A>G), 1: (c.563A>G/c.997C>T), 1: (c.563A>G/c.580T>C), 1: (c.563A>G/ c.379A>G)	2:(c.563A>G/ c.563A>G), 2: (c.584T>C/c.687G>T)
Genotype (protein variant)	NA	21: (p.Q188R/p.Q188R), 1: (p.Q188R/p.R333W), 1: (p.Q188R/p.F194L), 1: (p.Q188R/p.K127E)	2: (p.Q188R/p.Q188R), 2: (p.L195P/p.K229N)

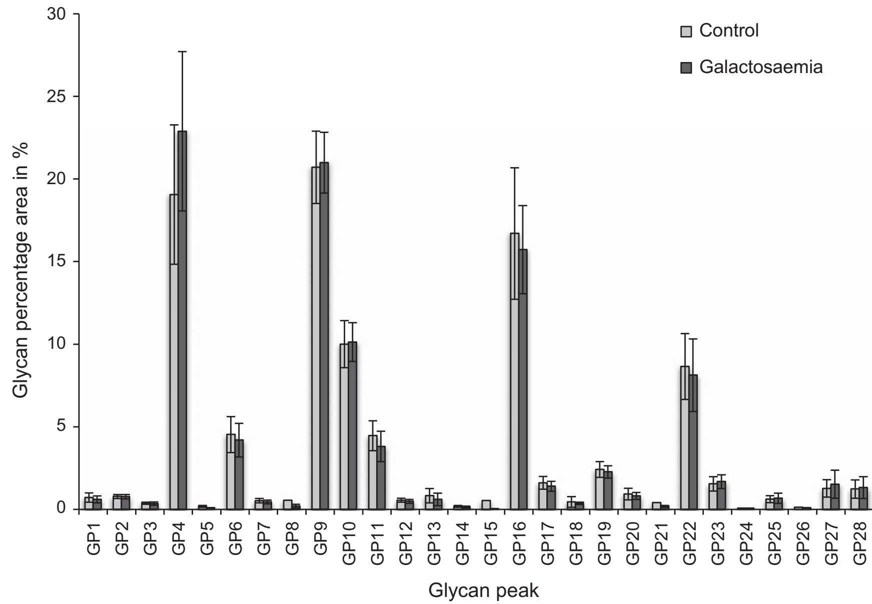
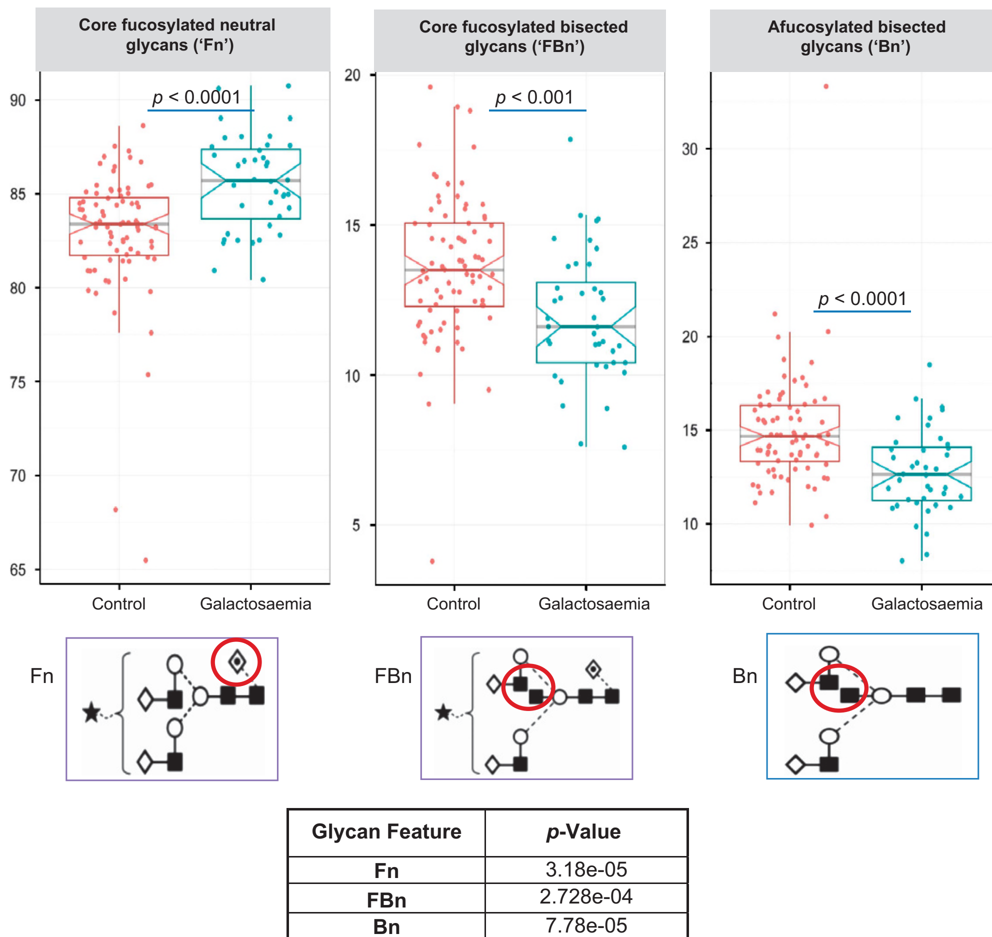
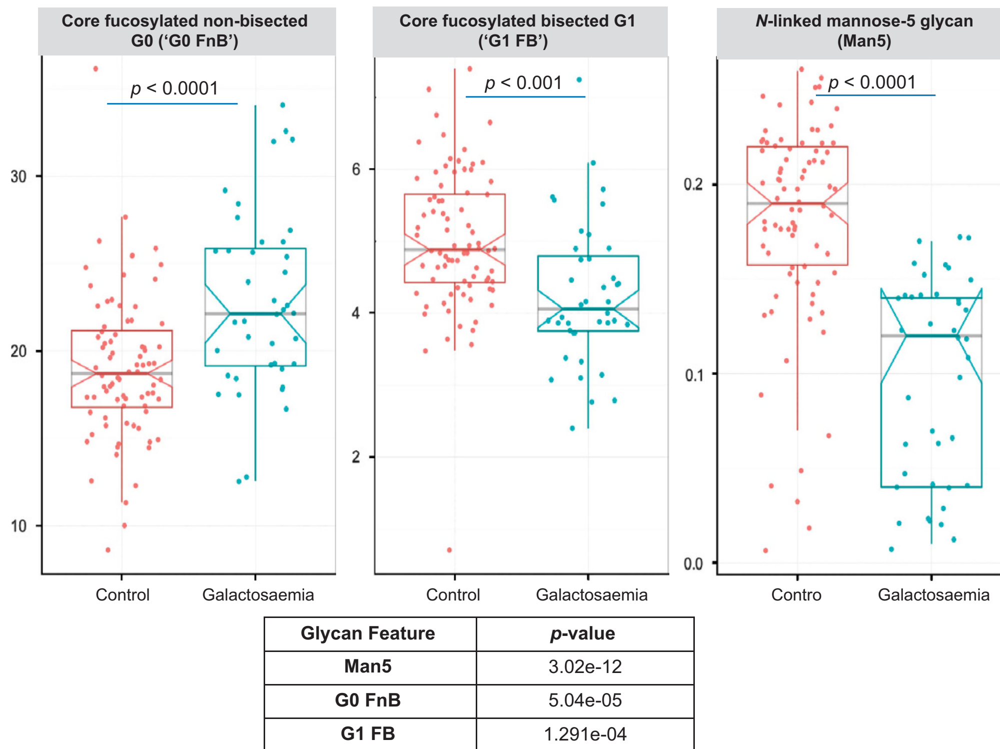


Figure 1 Area comparison of the 28 GPs from released undigested adult human serum IgG between galactosaemia patients and healthy controls. Samples were analysed by our recently reported improved method of automated HILIC-UPLC glycan analysis as described in Stöckmann *et al.*[32] GP, glycan peak number.



Glycan Feature	*p*-Value
Fn	3.18e-05
FBn	2.728e-04
Bn	7.78e-05

Figure 2 Box scatter plot representation of core fucosylation and bisected GlcNAcylation between the total galactosaemia group and controls. This depicts the increase in core fucosylated neutral glycans ('Fn'), the decrease in core fucosylated bisected glycans ('FBn') and the afucosylated bisected glycans ('Bn'), noted with an inverse relationship between total core fucosylation and bisecting GlcNAcs. The results are presented as a box scatter plot to show the spread of the individual values. Values for healthy controls are shown in red and total galactosaemia patients are shown in blue.



Glycan Feature	*p*-value
Man5	3.02e-12
G0 FnB	5.04e-05
G1 FB	1.291e-04

Figure 3 Box scatter plot representation of most significant changes that occur in individual glycans between the total galactosaemia group and controls. Data depict increase in core fucosylated non-bisected agalactosylated glycans ('G0 FnB'), decrease in fucosylated bisected monogalactosylated glycans ('G1 FB') and a very significant decrease in *N*-linked mannose-5 glycans (Man5). Data are given in a box scatter plot to show the spread of the individual values. Values for healthy controls are shown in red and total galactosaemia patients are shown in blue.

Oxford glycan annotation system

The specific *N*-glycans were annotated according to the Oxford notation system representing *N*-linked glycan composition and structure. The assignment of glycan structures by HILIC-UPLC in serum was performed by comparison with an updated GlycoBase 3.2 (http://glycobase.nibrt.ie/), as well as published assignments.[13,32] Assignment of the IgG glycans in each peak was based on the analysis in Pucic *et al.*[34]

Glycan feature statistical analysis

The analysis was performed using R software (www.r-project.org) with the R base package. Box plots were constructed using R package ggplot2.

TaqMan qPCR arrays

As a follow-up on the abnormalities of IgG *N*-glycan core fucosylation and sialylation observed, we sought to study the expression of a number of relevant *N*-glycan biosynthesis genes, which we have observed to be dysregulated in our earlier T lymphocyte expression study.[21] Therefore, we studied the gene expression in PBMCs from 32 adult classical galactosaemia patients (12 males and 20 females), and 10 adult controls. Briefly, PBMCs were isolated from whole blood within 2 h of collection using the BD Vacutainer cell preparation tubes (Fisher Scientific-362782, Fisher Scientific Ltd, Loughborough, UK), which allowed one-step isolation of living PBMCs. Total RNA was then extracted from PBMCs using the RNeasy Plus Mini kit (Qiagen-74134, Qiagen Ltd, Manchester, UK) and was retro-transcribed using the RT^2 First Strand kit (Qiagen-330401, Qiagen Ltd).

Custom-made ABI TaqMan Array plates (Applied Biosystems, Foster City, CA, USA) were constructed for four *N*-glycan synthesis target genes: *ALG9*, *MGAT1*, *MGAT3* and *FUT8* along with four housekeeping genes: *GAPDH*, *HPRT1*, *GUSB* and *ACTB*. qRT-PCR analysis was performed with these customised plates on a ABI PRISMR 7900 HT Sequence Detection System with 96-well standard thermal cycling block (Applied Biosystems). The relative gene quantification and statistical analysis was then performed using SDS Software Version 2.2.1 and DataAssist Software Version 3.0 with 2^ (-ΔΔCT) method (Applied Biosystems). Box plots were constructed using GraphPad Prism version 5.00 (GraphPad Software, San Diego, CA, USA).

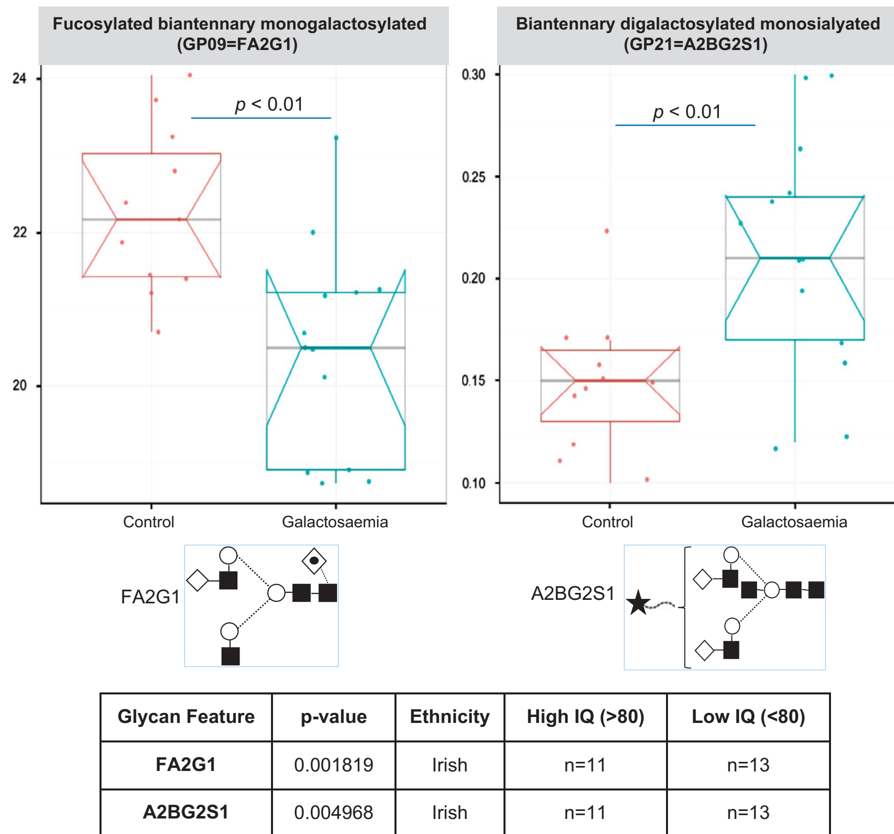
RESULTS

Expanded IgG *N*-glycan analysis of galactosaemia patients

Our recently improved high-throughput automated HILIC-UPLC methodology of the IgG *N*-glycan analysis[32,33] has allowed for more robust processing of total IgG, with improved GP resolution. In this study, we have observed 28 high-resolution IgG *N*-glycan peaks from released undigested adult galactosaemia patient serum compared with the healthy controls serum (Figure 1).

We have observed significant increases in IgG *N*-glycan core fucosylation, which is inversely related to glycans with bisecting GlcNAc. We observed that core fucosylated neutral glycans ('Fn') were significantly higher in galactosaemia patients ($P<0.0001$) than in controls. On the other hand, galactosaemia patients had significantly lower levels of core fucosylated bisected glycans ('FBn') and afucosylated bisected glycans ('Bn'), $P<0.001$ and $P<0.0001$, respectively (Figure 2).

Notable changes were also observed in individual glycans between the total galactosaemia group and controls. The most prominent finding was a significant decrease in *N*-linked mannose-5 glycans (Man5) ($P<0.0001$), as well as in core fucosylated bisected monogalactosylated ('G1 FB') glycans ($P<0.001$), and an increase in core fucosylated non-bisected agalactosylated ('G0 FnB') glycans ($P<0.0001$) was evident (Figure 3). A similar observation of increased levels of agalactosylated glycans in galactosaemia patients was evident in our earlier studies.[19,20]



Glycan Feature	p-value	Ethnicity	High IQ (>80)	Low IQ (<80)
FA2G1	0.001819	Irish	n=11	n=13
A2BG2S1	0.004968	Irish	n=11	n=13

Figure 4 Box scatter plot representation of possible correlation between IQ and glycosylation. Indicated GP with predominant structure as GP09 = FA2G1: fucosylated biantennary monogalactosylated, GP21 = A2BG2S1: biantennary digalactosylated monosialyated. Box scatter for statistically significant good neurological outcome or high IQ is shown in blue and red depicts poor neurological outcome or low IQ in adult galactosaemia patients, noted with an inverse relationship between core fucosylation and bisecting GlcNAcs.

We have observed a possible correlation between IQ and glycosylation in this study. IQ data were available for 24 Irish subjects over age 16 who all were homozygous for the *GALT* c.563A>G variant. We have observed notable significant correlations between IQ and the GPs with the predominant structures as fucosylated biantennary monogalactosylated (GP09 = FA2G1) glycans ($P<0.002$), and biantennary digalactosylated monosialyated (GP21 = A2BG2S1) glycans ($P<0.005$). An inverse relationship was noted between core fucosylation and bisecting GlcNAcs (Figure 4). Differentiation was made between a cut-off measured IQ of above or below 80.

Gene expression analysis reveals dysregulation of key *N*-glycan biosynthesis genes

As a follow-up to our previous studies, which reported abnormalities of IgG *N*-glycan core fucosylation and undersialylation and gene dysregulation in T-lymphocytes,[19,21] we studied the expression of a number of key *N*-glycan biosynthesis genes in PBMCs, which were dysregulated in our earlier T lymphocyte expression study.[21] The results of the large-scale validation study in treated adult patients of four *N*-glycan biosynthesis genes *(ALG9, FUT8, MGAT1 and MGAT3*) in freshly isolated PBMCs are represented in the heat map representation (Figure 5a). *ALG9* is significantly overexpressed ($P<0.001$; Figure 5b). This is similar to what we have previously reported in galactosaemia T lymphocyte preparations.[21] In addition, *MGAT1* gene expression is significantly decreased in galactosaemia samples ($P<0.01$; Figure 5b). *MGAT3* gene expression is decreased ($P=0.095$), and *FUT8* increased ($P=0.062$; Figure 5b).

DISCUSSION

It is well documented that, beyond the untreated neonatal period, analysis of whole serum or plasma *N*-glycome is largely unable to identify subtle differences in glycosylation between adults with galactosaemia.[6] The development of accurate and useful biomarkers, which reflect the disease pathogenesis is paramount, given the uncertain and often disappointing clinical outcome in treated galactosaemia individuals. We now have applied our advanced automated high-throughput HILIC-UPLC method to further study the IgG *N*-glycan abnormalities and affected pathways in an adult and adolescent cohort and controls.

Charlwood *et al.*[18] previously proposed that the *N*-glycan synthesis abnormalities observed in galactosaemia occur in glycoprotein processing and that partially processed *N*-linked glycans are diverted to alternative pathways, giving rise to bisected, or more highly branched structures. Furthermore, Sturiale *et al.*[17] showed that in untreated galactosaemia there is also a partial deficiency of whole *N*-glycans in serum transferrin associated with increased fucosylation and branching as seen in CDG-I, suggesting that there are joint *N*-glycan assembly and processing defects in untreated galactosaemia.

It is reasonable to propose that these ongoing abnormalities of *N*-glycosylation in treated patients will be replicated in many functionally relevant, systemic glycoproteins and glycolipids.

In this study, we have now identified a significant increase in core fucosylated neutral and agalactosylated serum IgG *N*-glycans, and a significant decrease in core fucosylated, afucosylated and monogalactosylated bisected glycans in treated galactosaemia patients, which we

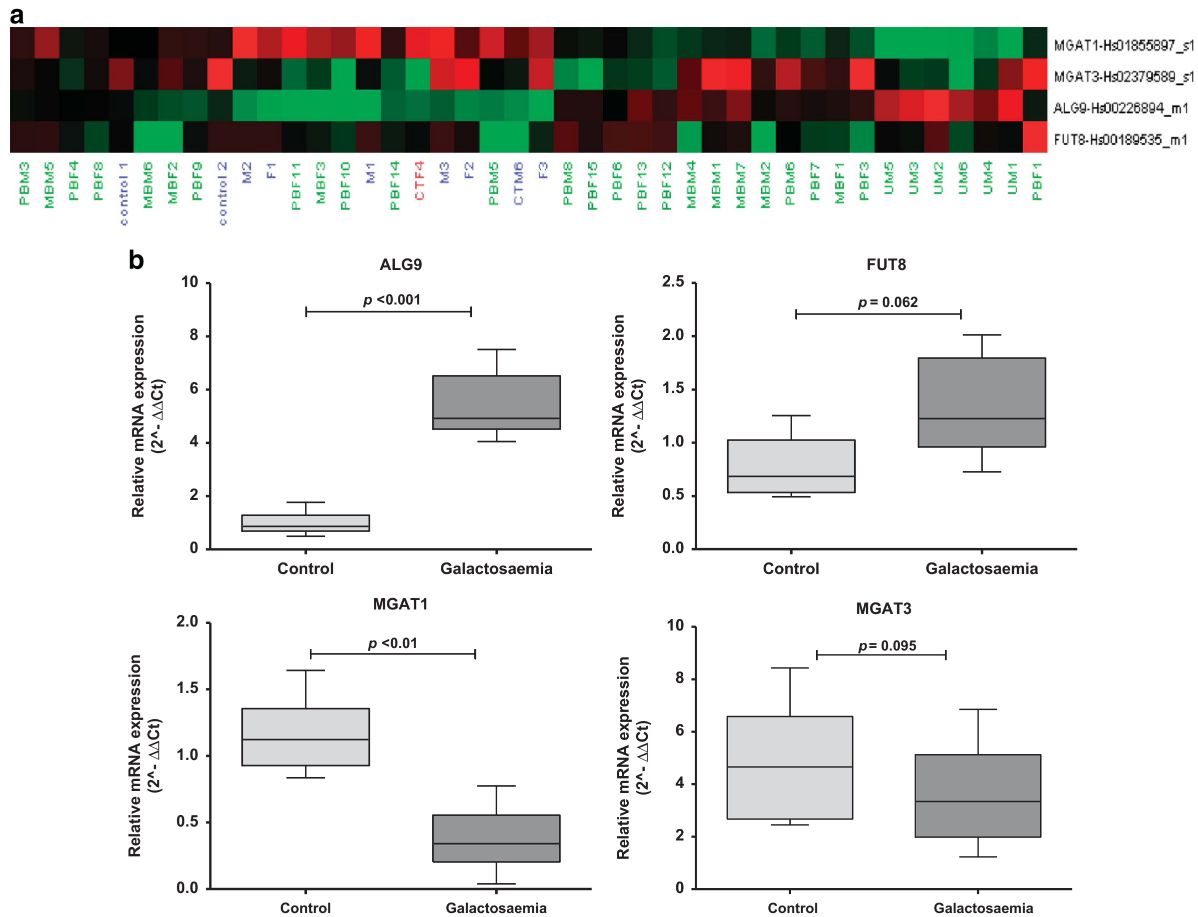


Figure 5 Gene expression profiling of 4*N*-Glycan biosynthesis genes in 32 adult classical galactosaemia patients and 10 adult controls using TaqMan PCR arrays. (**a**) Heat map representation of overall gene expression pattern, red indicates high gene expression and green indicates low. (**b**) Box plot representation of *ALG9, FUT8, MGAT1* and *MGAT3* genes, depicted with respective *P*-values between control and galactosaemia subject groups. Values for healthy controls are shown in the light grey box and galactosaemia patients are shown in the dark grey box.

consider may contribute to the ongoing pathophysiology and cell signalling abnormalities.[7,35] It is possible that the bisecting GlcNAcs could also influence glycan processing.

To summarise our current and previous observation, we present a schemata of the pathway illustrating the biosynthesis of lipid-linked oligosaccharides (LLO) in the endoplasmic reticulum (ER) (Figure 6a) and diversification of *N*-glycan biosynthesis in medial-Golgi (Figure 6b).[36] The green circles indicate observation from our previous T-cell microarray studies,[21] and the red circles indicate our observations in this study. From both studies, it is noted that several key mannosyltransferases in LLO biosynthesis in ER are dysregulated. The genes *ALG1, ALG2, ALG9, ALG8* and *RFT1* (*Flipase*) were noted to be significantly overexpressed in galactosaemia patients compared with controls, with a significant downregulation of the *DMP1* and *MGAT2* genes in our previous study of T-cell expression.[21] In this current study of PBMC expression, we have also noted a significant upregulation of *ALG9* and a significant downregulation of *MGAT1.* We have also noted an increase in *FUT8* expression and decrease in *MGAT3* expression in galactosaemia patients, and an associated decrease of biosynthesis of Man5 glycans, in the corresponding *N*-glycan analysis.

The ALG enzyme is involved in the addition of the seventh and ninth mannose to the growing *N*-glycan,[37] and thus essential for the formation of the initial oligosaccharide chain.[38] The dysregulation of the *ALG9* gene observed in galactosaemia patients thus suggests that there is at least partial disruption of the assembly pathway in treated adults. The disruption of assembly, resulting in protein misfolding, may cause subtle stresses to the cell, which may affect the function of the mannosyl-transferase enzymes, such as observed overexpression of *ALG1* and *ALG2* genes.

MGAT1, the product of which is essential for conversion of high mannose to hybrid and complex *N*-glycans, was noted to be significantly downregulated in treated adult galactosemia patients. The *MGAT1* gene encoding GlcNAc transferase I (alpha-1,3-mannosyl-glycoprotein 2-beta-*N*-acetylglucosaminyltransferase), adds GlcNAc to high-mannose sites an essential early step in producing all complex and hybrid *N*-glycans.[39,40] Of note, inactivation of the *MGAT1* gene in mouse oocytes was shown to impair oogenesis and mouse *MGAT1* knockouts were lethal, whereas conditional mutants show movement defects, tremors, paralysis characteristic of neurodevelopmental impairments and early death.[40–43] Also, of note, it was observed that null variants in *Drosophila* produced defects in locomotion and a reduced life span, whereas neuronal overexpression of *MGAT1* rescued shortened life span and increased life span.[44,45] We propose that physiologically relevant dysregulation of *MGAT1* in treated galactosaemia adults may be a consequence of decreased Man5 glycan bioavailability as substrate. This may contribute to the accumulation of high-mannose glycans in the pathway and to further *N*-glycan processing defects in treated galactosaemia patients.

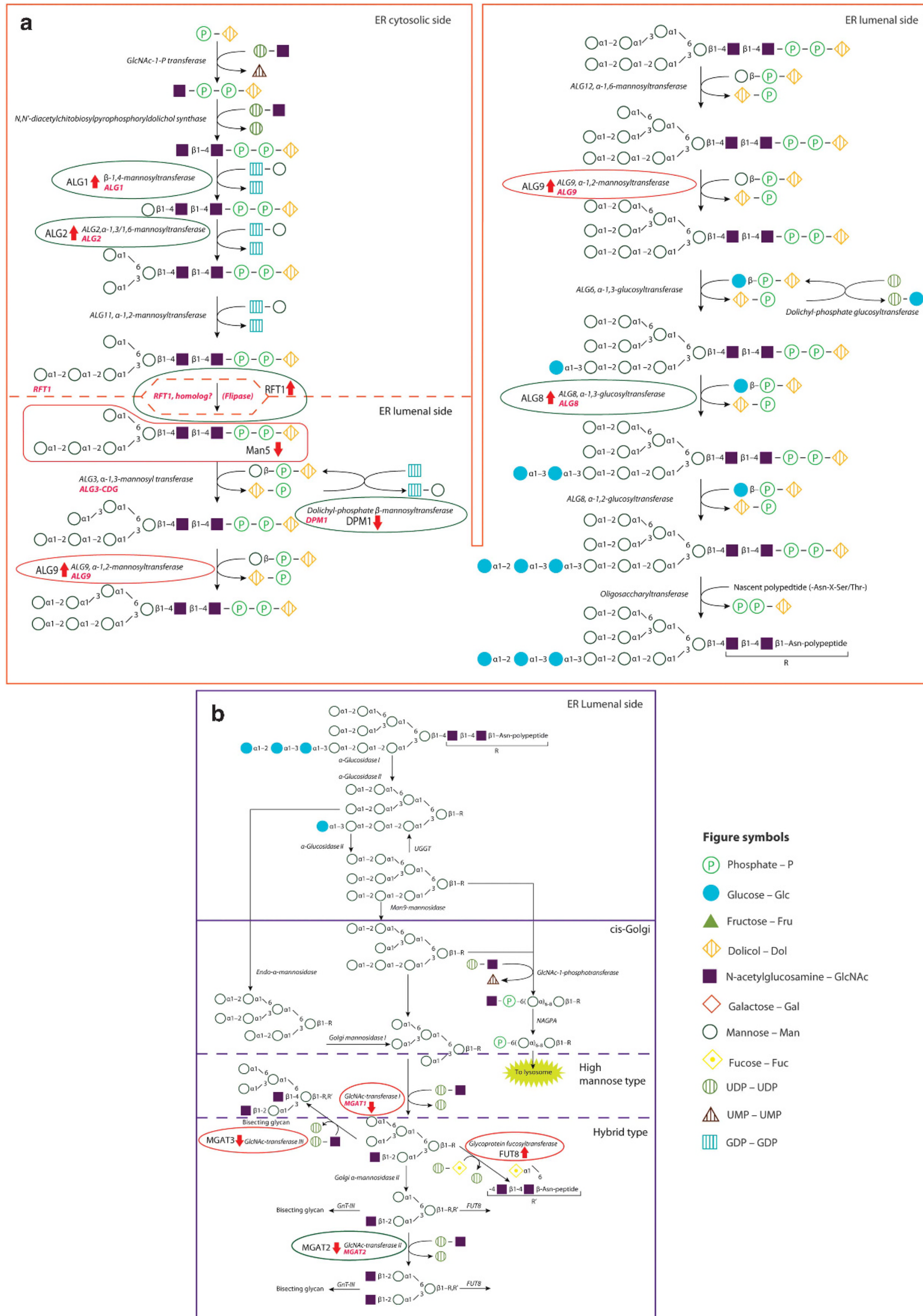


Figure 6 Biosynthesis of lipid-linked oligosaccharides in the ER (**a**) and remodelling of *N*-glycans in medial-Golgi (**b**) reconstructed from,[36] showing dysregulated pathway genes and glycans observed in these studies. The green circles indicate observation from our previous T-cell microarray studies.[21] The red circles indicate our observations from this study.

The expression of the genes: *FUT8* was noted to be increased and *MGAT3* decreased in galactosaemia patients. The dysregulation of *FUT8* (alpha-(1,6)-fucosyltransferase) and *MGAT3* (beta-1,4-mannosyl-glycoprotein 4-beta-*N*-acetylglucosaminyltransferase) may partly explain the increase in IgG core fucosylation and bisecting branching defects observed in this study. It was reported that the

dGALT null *Drosophila* disease model has deficient α1,3 fucosylation noted at the neuromuscular junction glycosylated synaptomatrix, with altered synaptic architecture and glycosylated synaptomatrix composition.[36]

From both our previous studies[21] and current observations, it is evident that, several key mannosyltransferases in LLO biosynthesis in ER and several vital *N*-acetylglucsoamine transferases in diversification of *N*-glycan biosynthesis in medial-Golgi are dysregulated. This suggests persistent processing and assembly defects in treated galactosaemia adults.

Of interest, we have also noted a statistical significant correlation between differences in IQ outcomes and the presence of core fucosylation and bisecting GlcNAcs abnormalities in these study subjects ($P<0.002$ for FA2G1 and $P<0.005$ for A2BG2S1).

As stated earlier, we have proposed that early-stage developmental, and also later ongoing disruption of glycosylation and related gene expression pathways may have a role in modifying the observed clinical outcome in classical galactosaemia. Abnormal glycosylation during prenatal galactose intoxication is proposed to affect long-term neurological development. These intriguing findings suggest that the variation in fucosylation and branching defects observed could have an influence in modifying the differences in outcome. These findings, taken in isolation, however, clearly cannot be used to predict outcome but may indicate individuals with 'favourable' or 'unfavourable' accessory glycosylation pathways, which may have influenced the tolerance to prenatal and perinatal galactose metabolite toxicity.

CONCLUSIONS

In summary, this study shows dysregulation of related IgG *N*-glycan biosynthetic pathway genes, *ALG9* and *MGAT1*, in treated galactosaemia adult patients and abnormalities of synthesis of Man5 glycans, bisecting GlcNAcs and core fucosylated glycans in treated adult and adolescent galactosaemia patients. These findings may have a role in the enigmatic pathophysiology of galactosaemia. Studies are required to elucidate the interplay of these genes on Man5 bioavailability and other metabolic factors. We suggest that IgG *N*-glycan profiling in conjunction with *ALG9* and *MGAT1* gene expression may potentially prove to be a sensitive and informative method of monitoring galactosaemia patients. These indices may act as indicators of variable clinical responses and offer new tools in determining individual treatment options and monitoring responses to new therapies.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

Funding for these studies was granted by the Irish Medical Research Charities Group/Health Research Board, Ireland (MRCG-HRB/CFFH (Temple St Childrens University Hospital). HS was supported by the EU FP7 Research Framework Program 'High Glycan' (grant reference no. 278535).

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