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Galactosemia, a Single Gene Disorder With Epigenetic Consequences

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ABSTRACT: Long-term outcomes of classic galactosemia (GAL) remain disappointing. It is unclear if the complications result mainly from prenatal-neonatal toxicity or persistent glycoprotein and glycolipid synthesis abnormalities. We performed gene expression profiling (T transcriptome) to characterize key-altered genes and gene clusters of four patients with GAL with variable outcomes maintained on a galactose-restricted diet, compared with controls. Significant perturbations of multiple cell signaling pathways were observed including mitogen-activated protein kinase (MAPK) signaling, regulation of the actin cytoskeleton, focal adhesion, and ubiquitin mediated proteolysis. A number of genes significantly altered were further investigated in the GAL cohort including *SPARC* (osteonectin) and *S100A8* (S100 calcium-binding protein). The whole serum *N*-glycan profile and IgG glycosylation status of 10 treated patients with GAL were compared with healthy control serum and IgG using a quantitative high-throughput analytical HPLC platform. Increased levels of agalactosylated and monogalactosylated structures and decreases in certain digalactosylated structures were identified in the patients. The persistent abnormal glycosylation of serum glycoproteins seen with the microarray data indicates persisting metabolic dyshomeostasis and gene dysregulation in "treated" GAL. Strict restriction of dietary galactose is clearly life saving in the neonatal period; long-term severe galactose restriction may contribute to ongoing systemic abnormalities. **(*Pediatr Res* 67: 286–292, 2010)**

Galactosemia (GAL) (McKusick 230400) caused by deficiency of galactose-1-phosphate uridyltransferase (GALT: EC 2.7.712) is associated with an impaired ability to metabolize galactose, derived from dietary lactose and endogenous sources (1). Galactose is normally converted to glucose-1-phosphate and metabolized to release energy or alternatively galactose may be metabolized to UDP-galactose (UDP-GAL) and its derivatives, which serve as key substrate donors for the biosynthesis of glycoproteins and glycolipids.

The acute symptoms of GAL include poor feeding, vomiting, liver dysfunction, hypotonia and lethargy, cataracts, and predisposition to sepsis. Long-term treatment, consisting of a severe restriction of dietary galactose is life saving in the newborn and avoids often fatal liver disease. However, long-term follow-up has shown that, despite a strict diet, most patients develop abnormalities such as intellectaul, motor and language development and hypergonadotrophic hypogonadism in females (1–4).

GAL when untreated is thought to simulate a number of the inherited genetic defects of *N*-glycosylation as there are apparent abnormalities noted in glycosylation of a number of glycoproteins such as transferrin and FSH in affected females (1,5–7). Untreated patients with GAL have been shown to have truncated glycans deficient in sialic acid and galactose suggesting defective *N*-glycan assembly and processing (7,8).

There are a number of possible pathophysiological mechanisms for the complications of treated GAL. GALT deficiency may lead to defective synthesis of glycoproteins and galactolipids critical for normal myelin formation possibly as a result of defective transfer of galactose from UDP-GAL and low UDP-Gal concentrations. Galactose-1-phosphate also competitively inhibits UDP-glucose pyrophosphorylase leading to a reduction in UDP-glucose/galactose content in GALT-deficient cells (9). GALT-deficient fibroblasts responded to galactose challenge by up-regulating a set of genes characteristic of endoplasmic reticulum (ER) shock and unfolded protein response (10) Deficiencies of glucose/galactose, crucial glycosyl donors could impair protein glycosylation reactions and trigger ER stress (10).

We recently compared outcomes in siblings with up to 25 y of treatment from our center where there is a relatively high incidence of GAL. Outcomes were disappointing with a high incidence of decreased intelligence quotient (IQ) and neurologic complications, despite strict galactose restriction with no correlation with biochemical control (4). These findings have led us to consider that treated patients with GAL still have persistent systemic abnormalities of *N*-glycosylation, which might cause abnormal glycoprotein functioning and protein folding (in the CNS and systematically).

We sought to study the contemporary gene expression analysis (microarray analysis) in a subset of our patients with GAL to include two cases with good neurologic outcomes and two with poorer outcomes to identify possible epigenetic

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Abbreviations: GAL, galactosemia; **KEGG,** kyoto encylopaedia of genes and genomes; **G0,** agalactosylated; **G1,** monogalactosylated; **G2,** digalactosyalted

effects determining variable outcomes. We have identified multiple aberrations in gene expression in these patients compared with controls in important cell survival pathways such as the ubiquitin-mediated proteolysis (UPR), Ca^{2+} signaling, RAS and mitogen-activated protein kinase (MAPK) signaling, actin cytoskeleton, glycosylation, and cell adhesion.

We have also performed biochemical analysis of current glycosylation status of *N*-linked serum glycoproteins, in two of the earlier patients and in a second cohort of adult patients with classical GAL.

METHODS

Patients with GAL. The characteristics of the four study subjects for the microarray analysis including the two who had glycoprotein analysis performed are listed in Table 1. Patients 1 and 2 display severe neurodevelopmental sequelae of GAL, whereas patients 3 and 4 display normal intellect and executive functioning. Patients 1 and 3 are siblings. Three subjects are adults of Irish origin and have severe GAL diagnosed in the neonatal period with homozygozity for the null allele *Q188R*. One subject is a 3-y-old African boy who has a milder variant of GAL (homozygous for *S135L*), who was late diagnosed at age two and has cognitive impairment (11). Five milliliters of whole blood was collected for lymphocyte extraction from patients and a control group.

We performed normal phase (NP) HPLC *N*-glycan analysis for two of the patients described in Table 1 as earlier, (patients 1 and 4) and a second cohort of eight patients with classic GAL, seven adults and one teenager. All are homozygous for the severe mutation *Q188R* and have favorable outcomes with mean IQ scores >80 and remain on galactose restriction of <150 mg/d, with the exception of patient 1 with a current IQ of 40 and patient M who had self liberalized his diet to ~4 g galactose/d (approximately half of the normal adult galactose intake). Ethics approval for this study was obtained from the Ethics Committee of the Children's University Hospital, Dublin. All patients and controls gave full informed written consent before enrolment.

Microarray analysis. T lymphocytes were extracted from whole blood within 2 h of collection using enrichment columns (R&D Systems). RNA was extracted and prepared according to the Affymetrix guidelines for Human Genome U133 Plus 2.0 microarrays. Microarrays were scanned using an Axon scanner with images analyzed using GenePix image software. The resulting CEL files were analyzed using RMAexpress software and genes with at least 1.5-fold expression change ($p < 0.05$) were shortlisted for further analysis. Two comparisons were performed; expression profiles between normal and patients with GAL in addition to between patients with mild and severe GAL. Control samples were derived from three healthy adult controls. Shortlisted genes were further studied using DAVID EASE (12) (www.david.abcc.ncifcrf.gov). Genesets were curated from biochemical pathway data available from DAVID EASE and the kyoto encyclopedia of genes and genomes (KEGG) (www.genome.jp/kegg). KEGG pathways with two or more members represented in the gene lists of dysregulated genes were shortlisted.

Reverse-transcription and real-time polymerase chain reaction. To validate our microarray findings, we verified the levels of three dysregulated genes (*RGS10*, *SPARC*, and *S100A*) in addition to one housekeeping gene (*GAPDH*).

Total RNA (2 μg) from the patients and controls was retrotranscribed using Super Script System (Invitrogen) and quantitative real-time PCR was performed using a rotor gene system (Corbett research). For every sample, the genes were amplified in triplicate in the same run. PCR results between the four index patients and controls were compared and the results were analyzed using the *t* test. In addition, the quantitative real time PCR experiment was independently validated from RNA samples derived from three of the second patient cohort used for the glycobiology studies.

Methods for NP HPLC glycan analysis. A high-throughput analytical platform was applied to whole serum and purified IgG *N*-glycans, using previously described methods (13). This entailed sample immobilization, enzymatic *N*-glycan release, fluorescent labeling with 2-aminobenzamide (2-AB), and NP HPLC profiling with database interrogation. IgGs were then isolated from patient serum and the *N*-glycans were then processed and labeled with 2-AB before injection onto the HPLC. Undigested serum IgG *N*-glycans initially analyzed revealed differences in the G0/G1 and (G0/G1)/G2 ratios between patient samples and a pooled control sample from >20 healthy individuals. The IgG-derived *N*-glycans were then digested with a *Arthrobacter ureafaciens* sialidase (α2–3,6,8 sialic acid) (ABS) and bovine kidney α-fucosidase (α1–2,6,3,4) (BKF) to remove sialic acids and core fucose, respectively, to allow a more accurate calculation of the G0/G1 and (G0/G1)/G2 ratios.

RESULTS

Microarray analysis. Comparing the dataset of patients with GAL with controls, 9797 genes in total were up-regulated, and 9896 genes were down-regulated involving a number of major pathways. As seen in Figure 1, the four most represented pathways included the MAPK signaling pathway, regulation of actin cytoskeleton, focal adhesion, and ubiquitin mediated proteolysis.

Sixteen genes were up-regulated at least 100-fold in T lymphocytes from patients with GAL in comparison with control. The top genes up-regulated and down-regulated, respectively, are noted in Table 2. When comparing the profiles of patients with poor *versus* good outcomes, 212 genes were noted to be up-regulated in the severe outcome group, and 232 genes were down-regulated (Table 3).

Table 1. *Patient characteristics*

Patient demographics	Patient 1*‡	Patient 2	Patient 3*	Patient 4
CNS severity	Severe	Severe	Mild	Mild
Age at time of study	26	3	27	25
Age at diagnosis	Neonate	1	Neonate	Neonate
Gender	Female	Male	Male	Female
Nationality	Irish	Nigerian	Irish	Irish
Genotype	Q188R/Q188R	S135L/S135L	Q188R/Q188R	Q188R/Q188R
GALT activity†	<0.5	<0.5	<0.5	<0.5
Growth <10th centile	Yes	Yes	No	No
Complications/ outcome	Severe developmental delay, IQ 40 verbal dyspraxia, ataxia hypergonadotrophic hypogonadism	Moderate-severe global developmental delay, significant verbal dyspraxia. Cataracts noted 3 y	Normal IQ	Mild speech and language delay as a child. Normal IQ hypergonadotrophic hypogonadism
MRI CNS imaging	Prominent cerebellar sulci, cerebral atrophy	Poor and delayed myelination (1.5 y)	Normal	

Patients 1 and 2 display severe developmental and intellectual complications and patients 3 and 4 have normal intellectual outcomes.

* Designates siblings.

† GALT expressed as μmole subc/H/gHb, galactose-1-phosphate.

‡ Mother on limited galactose intake during pregnancy.

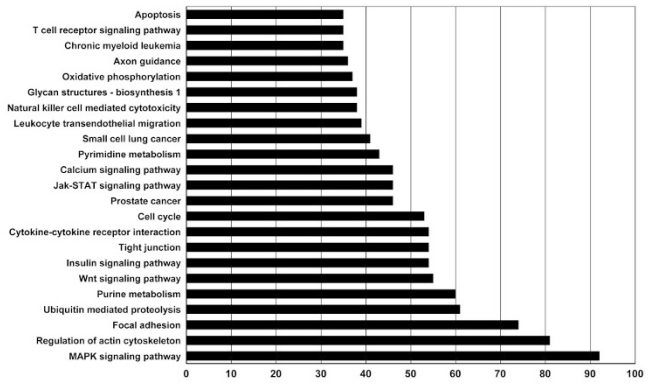


Figure 1. KEGG pathways where at least two gene members are dysregulated.

Table 2. *Differential expression of genes (up-regulated/down-regulated) in total group of galactosemic patients in comparison with controls*

NCBI number	Gene title	Gene symbol	Fold increase/ decrease
Up-regulated			
NM_001004	Ribosomal protein, large P2	*RPLP2*	324.0
NM_004126	Guanine nucleotide binding	*GNG11*	205.2
NM_004657	Serum deprivation response	*SDPR*	165.8
NM_002925	Regulator of G protein signalling	*RGS 10*	159.1
NM_002213	Integrin, beta 5	*ITGB5*	147.3
NM_053031	Myosin light chain kinase	*MYLK*	146.6
NM_003973	Ribosomal protein L14	*RPL14*	139.2
NM_003118	Secreted protein acidic	*SPARC*	138.7
NM_002964	S100 calcium binding protein	*S100A8*	122.3
NM_130782	Regulator of G protein signalling	*RGS18*	121.7
Down-regulated			
NM_004574	Septin 4	*SEP–04*	85.8
NM_024092	Transmembrane protein 109	*TMEM109*	49.2
NM_001037500	Defensin, beta 124	*DEFB124*	40.7
NM_152603	Zinc finger	*ZNF567*	27.1
NM_175066	Dead box polypeptide	*DDX51*	26.7

Table 3. *Differential expression of genes (up-regulated/down-regulated) in galactosemic patients with poor outcomes in comparison to favourable outcomes*

NCBI number	Gene title	Gene symbol	Fold increase/ decrease
Up-regulated			
NM_020666	CDC-like kinase 4	*CLK4*	4.4
NM_017664	Ankyrin repeat domain 10	*ANKRD10*	2.9
NM_005520	Heterogeneous nuclear ribonucleoprotein H1(H)	*HNRNPH1*	2.9
NM_024941	Hypothetical protein FLJ13611	*FLJ13611*	2.7
NM_053024	Profilin 2	*PFN2*	2.7
NM_000598	Insulin-like growth factor binding protein 3	*IGFBP3*	2.6
NM_05278	Glycoprotein M6B	*GPM6B*	2.6
NM_016730	Folate receptor 1 (adult)	*FOLR1*	2.5
Down-regulated			
NM_152545	RasGEF domain family, member 1B	*RasGEF 1B*	5.9
NM_025217	UL16 binding protein 2	*ULBP2*	4.6
NM_173171	Nuclear receptor subfamily 4, group A,	*NR4A2*	4.1
NM_015014	RNA binding motif protein 34	*RBM34*	4.1
NM_003745	Suppressor of cytokine signalling 1	*SOCS1*	4.0

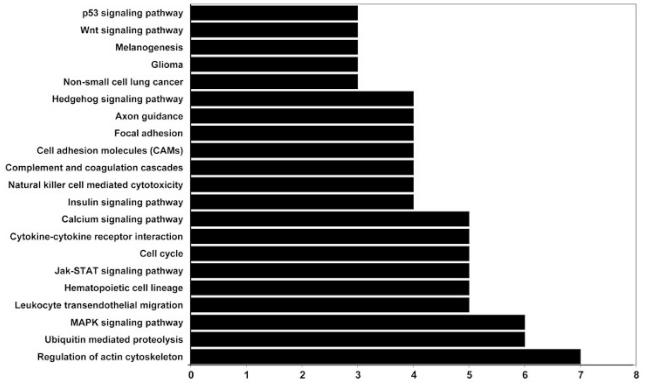


Figure 2. KEGG pathways where at least two gene members are dysregulated in patients with GAL with poor neurologic outcomes in comparison with favorable outcomes.

The corresponding KEGG pathway analysis comparing poor *versus* favorable neurologic outcomes showed dysregulation of multiple pathways including components of the actin system, MAPK signaling, cell cycle, calcium signaling, natural killer cell-mediated cytotoxicity, cell adhesion molecules, and the hematopoietic cell lineage pathways (Fig. 2).

To confirm the gene array expression data, real-time quantitative RT-PCR was performed for three genes; *SPARC*, *S100A8*, and *RGS10* (listed in Table 2). The gene expression was independently validated in three RNA samples derived from patient with GAL samples (not used in the primary analysis). All three genes were independently confirmed to be up-regulated in GAL lymphocytes in comparison with normal (Fig. 3).

Assessment of* N-*glycan galactose status. Figure 4 shows the main pools of structures present in a NP HPLC profile of total *N*-glycans released from pooled serum (adapted from reference 13). Figure 5 represents a typical whole serum *N*-glycan profile for two treated patients with GAL (one with a good outcome and one with a poor neurologic outcome) in comparison with a pooled healthy control sample. Figure 6 outlines the nomenclature system used by the NIBRT Dublin-Oxford Glycobiology Lab (14). The increased peak areas in the profiles for the patients with GAL are consistent with a lower percentage of galactose incorporation into biantennary serum glycoproteins in comparison with control. These *N*-glycan alterations were common across the GAL cohort and have generally been reported previously (7,8).

For the severely affected patient (1), the total serum *N*-glycan profile contains increased levels of G1 biantennary structures compared with control. However, this sample also showed differences in comparison to the other patient with

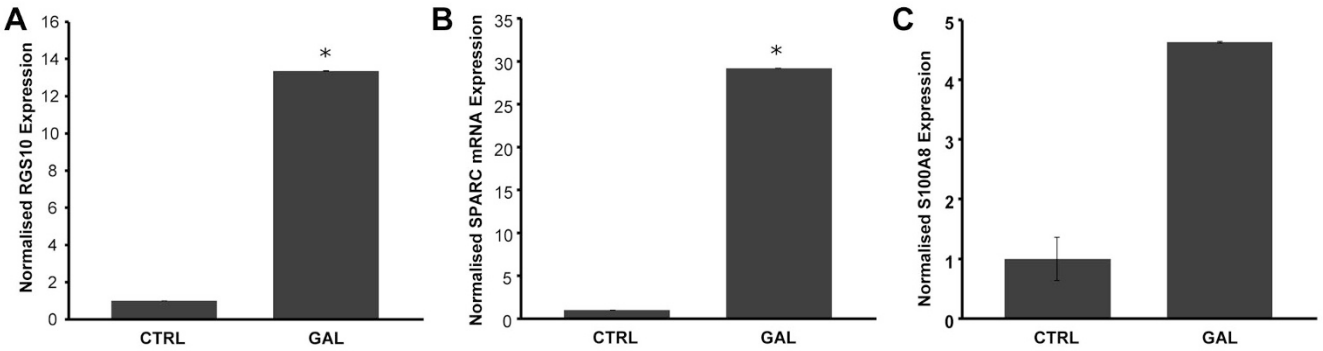


Figure 3. Verification of expression microarray data by quantitative real-time PCR. The graph represents the fold differences on expression level of the selected genes in RNA samples from the four index cases and three independent patients with GAL. *$p < 0.05$. (*A*) RGS10, (*B*) SPARC, (*C*) S100A8.

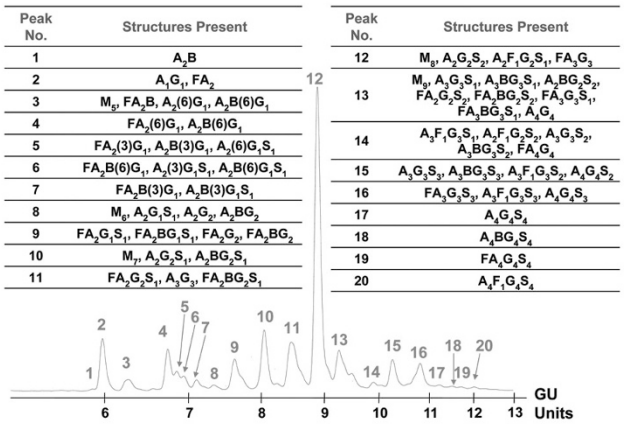


Figure 4. Represents the *N*-glycan profile of total serum glycoproteins released from a pooled serum sample. The glycan structures present in each of the pools are shown.

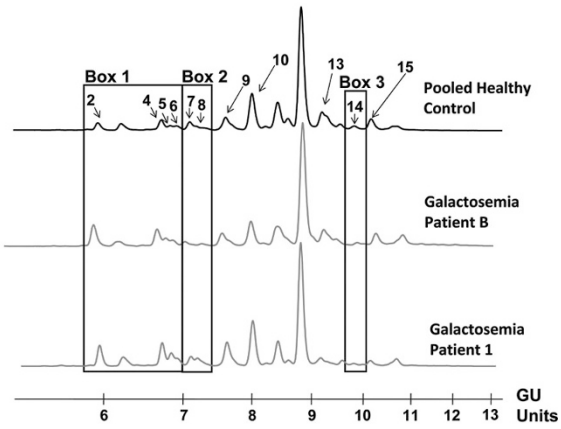


Figure 5. Illustrates a comparison of the total serum glycome from a healthy control, a representative patient with GAL and the patient with the most severe outcome (patient 1). Areas of particular interest are highlighted with boxes and arrows. *Box 1* shows an increase in peaks 2, 4, 5, and 6 in both the patients with GAL relative to control. A decrease was shown in peaks 7 and 8 (*Box 2*) and peak 14 (*Box 3*) in the treated GAL profiles when compared with control. Patient 1 shows unique alterations in particular glycan pools such as increases in peaks 9 and 10 as well as a decrease in peaks 13 and 15 relative to both control and the overall GAL cohort.

GAL serum samples, including increased levels of A2G1, FA2G1 A2G2S1, and the bisects of some of these structures. This patient also has a decrease in the glycan pool containing

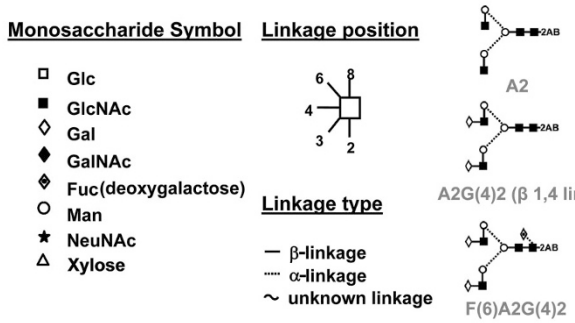


Figure 6. Outlines the nomenclature system used by the NIBRT Dublin-Oxford Glycobiology Laboratory (see Ref.14 for structure designation).

large structures such as A3G3S3 (pool 15) relative to both the control samples and other GAL samples and also loss of core fucosylated A2G2S2 structures (pool 13).

We investigated the G0/G1 and (G0/G1)/G2 ratios between serum IgG purified from the GAL cohort and a pooled control sample (13). A representative trace for human serum IgG from a patient with GAL is shown in Figure 7. There was an increase in G0/G1 ratios between the GAL and control traces (Fig. 8), which was found to be significantly different (average fold increase of 1.71 ± 0.34 SD; *t* test $p = 0.02$). However, the (G0/G1)/G2 ratio was not significantly different (*t* test $p = 0.94$) when the GAL cohort was compared with the pooled control.

Figure 7 indicates a number of pools in the IgG glycan profile containing both mono- and di-galactosylated structures. To allow for a more accurate measurement of the G0/G1 and (G0/G1)/G2 ratios, exoglycosidase digestions were carried out on the purified IgG *N*-glycans using ABS and BKF to remove terminal sialic acid and core fucose from these samples. Figure 9 shows a representative ABS BKF digestion profile for a patient with GAL in comparison with a pooled control sample. Again, there is a significant increase in G0 containing peaks in the GAL set and a decrease in G2 peaks. The G0/G1 and (G0/G1)/G2 ratios from the IgG *N*-glycan digestion products were then determined for the GAL and control cohorts (Fig. 10). The difference between the two groups was significant (*t* test $p = 0.003$ and 0.004, for G0/G1 and G0/G1/G2 ratios, respectively). Of note, patient M, who has self-liberalized his diet, and also patient 1 were outliers

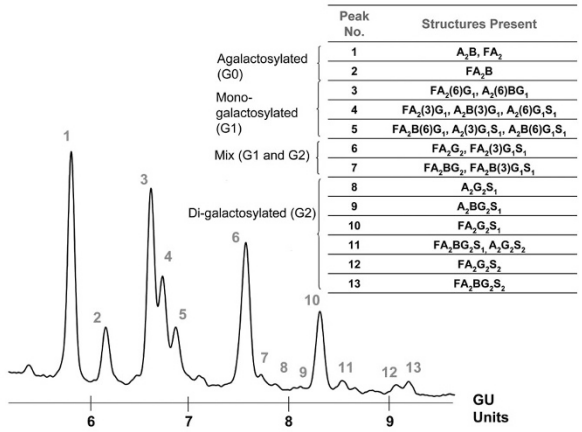


Figure 7. Representative chromatograph for human serum IgG glycans from a patient with GAL with the glycan structures listed for each designated pool. A number of pools in the undigested IgG profile contain both mono- and di-galactosylated structures.

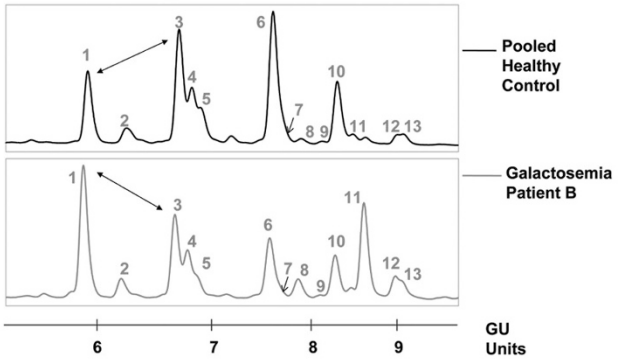


Figure 8. Representative chromatographs for undigested IgG-derived *N*-glycans from the pooled control sample and a patient with GAL. The GAL sample demonstrates an increase in agalactosylated IgG *N*-glycans compared with control (indicated by arrows).

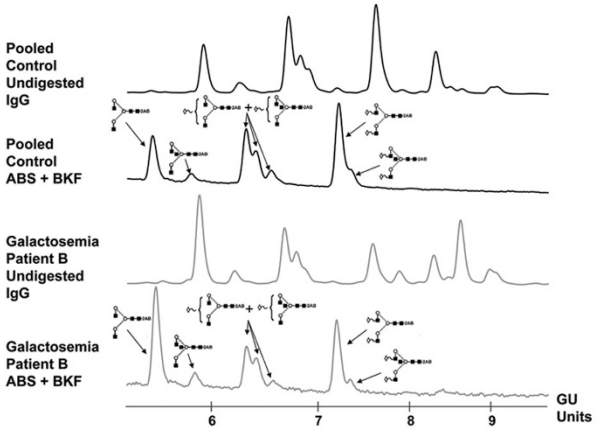
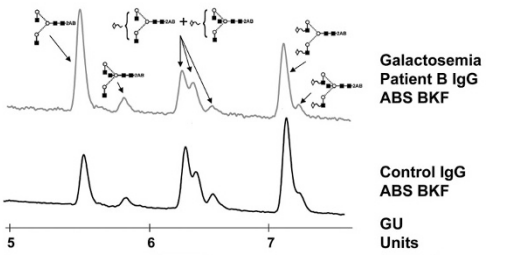


Figure 9. Representative ABS BKF digestion profile for a patient with GAL *vs* a pooled control sample. Again, there is an increase in G0 containing peaks in the GAL set and a decrease in G2 peaks.

with the lowest (G0/G1)/G2 ratio of the GAL set. This may indicate improved IgG galactosylation status in patient M. However, this finding is somewhat paradoxical in the context of the more abnormal total serum glycan profile seen for patient 1. The relatively low (G0/G1)/G2 ratio found in patient



	Pt. A	Pt. B	Pt. C	Pt. D	Pt. M	Pt. N	Pt. O	Pt. P	Pt. Z	Pt. 1	Con	Con
On Diet?	Yes	Yes	Yes	Yes	No	Yes	Yes	Yes	Yes		-	-
G0	27.2	40.5	46.5	35.0	20.9	34.8	30.5	32.5	33.3	25.1	19.7	20.2
G1	36.5	30.5	29.2	29.1	27.1	33.2	26.1	33.6	36.8	40.4	39.7	40.0
G2	36.4	29.0	24.3	35.8	51.9	32.0	43.4	33.9	29.9	34.5	40.5	39.8
G0/G1	0.7	1.3	1.6	1.2	0.8	1.0	1.2	1.0	0.9	0.6	0.5	0.5
(G0/G1)/G2	0.02	0.05	0.07	0.03	0.01	0.03	0.03	0.03	0.03	0.02	0.01	0.01

*Patient M has begun self-liberalisation of his diet

Figure 10. G0/G1 and (G0/G1)/G2 ratios from the IgG *N*-glycan digestion products for the GAL and control samples.

1 may reflect the increased levels of biantennary G1 and G2 structures and concomitant decrease in large branched structures detected in the total serum profile relative to the other patients with GAL (Fig. 5).

DISCUSSION

In this study, we have demonstrated substantial differential gene expression in T lymphocytes in a small cohort of patients with GAL in comparison with controls and also have demonstrated significant differential gene expression between patients with GAL with satisfactory and poor neurologic outcomes. Our results indicate that GAL is associated with common and precise gene expression alterations affecting multiple metabolic pathways.

The array data that are currently presented represent the expression profiles of patients with GAL on a galactose restricted diet in T lymphocytes and do not represent the earlier life events or untreated GAL.

The process of glycosylation modulates interactions of receptors and ligands with themselves, coregulatory molecules and distinct membrane domains of intact cells, thus altering signal transduction. Variation in glycolipid function regulates the activation of various cell-surface growth factor receptors by mechanisms that also modulate ligand binding and receptor dimerization (15).

Comparing patients with GAL with normal controls, we have identified major systemic signaling pathway dysfunctioning in these patients, to include aberrations of the MAPK signaling pathway, regulation of the actin cytoskeleton, focal adhesion, and cytokine-cytokine receptor interaction. UPR is also substantially affected, for example *USP 42* is 10-fold up-regulated. Disturbed UPR is well recognized to have a significant role in many neurodegenerative diseases, (*e.g.* Alzheimer's disease, Parkinson's disease) (16,17).

Figure 2 illustrates the comparative expression pattern between patients with GAL with favorable *versus* poor outcomes, indicating affected pathways to include MAPK signaling, regulation of actin cytoskeleton, the calcium signaling pathway, cell adhesion molecules in addition to the hematopoietic cell lineage, and the cell cycle. Table 2 illustrates the

top 10 genes up-regulated in the total GAL group. Two regulators of G protein signaling were significantly up-regulated in the total GAL group, *RGS10* and *RGS18* (159- and 121-fold increased, respectively). The RGS family are known to negatively impair signaling pathways involving transmembrane receptors and G proteins including MAPK activation by mammalian G-protein-linked receptors (18,19). In relation to the dysregulation of calcium signaling noted in our model, of note, *RGS10* has been shown to be a key regulator of Ca^{2+} oscillations (20) and in an *in vitro* system over expression of *RGS18* attenuated inositol phosphate production via G-alpha (q) in response to stimulating the angiotensin receptor (21). Slepak *et al.* (10) have noted altered calcium homeostasis in a GAL cell line when exposed to galactose with decreased free Ca^{2+} release from ER storage proposed to also operate through this pathway.

Expression of *S100A8* (calprotectin subunit), a member of the S100 family of Ca^{2+} binding proteins accepted as a marker of inflammation, was markedly increased (122-fold). With S100A9, S100A8 is the major calcium and zinc binding protein of phagocytes and keratinocytes and has a critical factor in the innate immune response to infection (22).

Integrin beta 5 is 147 times up-regulated in patients with GAL in comparison with controls with also up-regulation of *ITGB1* and *ITGB1BP1*. Integrins are transmembrane receptors (glycoproteins) required for extracellular matrix and cell surface proteins, with functions including attachment and spreading, cytosolic signal cascades to promote cell migration, survival, proliferation, and differentiation (23). Integrin-mediated cell adhesion regulates gene expression via the activation of transcriptor factors, *e.g.* phosphatidylinositol 3-kinase, and may play a role in Wnt signaling pathways and expression of growth factor receptors (24).

SDPR, serum deprivation protein response (implicated in endocytosis, transcytosis, and cell signaling) was shown to be markedly up-regulated (25).

Epidermal growth factor (*EGF*) was 27-fold increased in the total patient group, whereas expression of *EGFR* a heavily glycosylated protein was decreased 4-fold. EGFR significantly affects MAP kinase signaling. These findings may relate to the growth retardation observed in some patients with GAL. *EGFR* expression has previously been shown to be decreased in a GALT-deficient cell line, proposed to result from a change in EGFR protein synthesis or turnover (10).

SPARC or osteonectin, a secreted Ca^{2+} binding glycoprotein important to bone calcification was up-regulated 138-fold. *SPARC* null mice develop cataracts and severe osteopenia (26). Up-regulation of this gene may be secondary to the osteopenia noted in clinically treated patients with GAL (1).

Table 2 illustrates the top genes significantly down-regulated in patients with GAL in comparison with controls. The gene most highly dysregulated (decreased 85-fold) is *Septin 4*. Septins are a conserved group of GTP-binding and filament-forming proteins with diverse cellular functions including membrane dynamics, cytoskeleton reorganisation, polar determination, vesicle trafficking, exocytosis, and apoptosis and have been implicated in human neurodegenerative disease states such as Alzheimer's disease, Down syndrome, and in juvenile Parkinson's syndrome (27). Septins also play a significant role in innate host immunity. Microorganisms require interactions with the cytoskeleton, of possible relevance to the predisposition to *Escherichia coli* sepsis in untreated GAL neonates (28).

DEFB124, defensin beta was also down-regulated 40-fold. Defensins are antimicrobial peptides active at cell surfaces with cytokine activity, important in innate host immunity (29).

Table 3 illustrates a number of genes with significant differences in expression between the severely affected and milder outcome patients.

Galactosylation is abnormal in patients with GAL and may play a role in the CNS manifestations of GAL by means of (1) impaired galactosylation of cerebrosides and glycoproteins in the brain (30) and (2) a reduction in UDP-glucose/galactose contents in GALT-deficient cells (9).

The *N*-glycan analysis of whole serum from these patients highlighted a general increase in the levels of G0 and monogalactosylated G1 structures and a decrease in G2 and sialyated structures when compared with a control sample. Analysis of the serum IgG *N*-glycans from these patients indicated that levels of the (G0/G1)/G2 ratio were higher in the GAL set and an individual patient who had already begun self-liberalization of his diet had a (G0/G1)/G2 ratio, which was within control levels with less pronounced glycan abnormalities compared with the other patients, consistent with improved glycosylation status. It should be noted that these differences may also correspond to altered levels of serum glycoproteins and that the site occupancy of IgG has not been investigated in this study.

In this study, we have used IgG *N*-glycosylation as a model for aberrations in glycosylation in patients with GAL. Our findings of increased levels of G0 structures associated with IgG potentially have a number of systemic implications. Alterations in the IgG associated *N*-glycans such as decreased galactosylation leading to decreased sialylation can result in localized conformational changes which can in turn affect the interaction between IgG-Fc and other molecules. Terminal galactose residues on IgG are important for maintaining the half life and turnover rate of IgG by allowing it to bind efficiently to FcRn of relevance to autoimmune conditions such as rheumatoid arthritis and activation of the complement cascades and immune responses (31,32).

Given the systemic implications of altered glycosylation of IgG, it is likely that patients with GAL exhibit a number of altered glycoproteins, with distinct functional consequences, which could impact on a wide range of physiologic pathways. In support of this hypothesis, the whole serum *N*-glycan profile from the GAL cohort may contain G0-, G1-derived structures from incompletely processed glycans from proteins other than IgG (Fig. 5 *Box 1*).

Our preliminary serum glycoprotein data are consistent with differing levels of abnormalities of glycoprotein processing combined with changes that suggest an assembly defect (see patient 1 in Fig. 5, the most severely affected patient on strict galactose restriction). It is noteworthy that the most "normal" profile in relation to the incorporation of galactose is in a patient on a liberalized diet. Although it is presumed that

the main determinant of neurologic outcome related to glycoprotein and glycolipid synthesis is established during the first few years of life, it is interesting to note that there is a persistent abnormal profile in the most severely affected adult patient. From the total serum and IgG-derived *N*-glycan profiles seen for this patient, it is not possible to identify a specific isolated *N*-glycan biosynthetic pathway defect because the contributing causes are expected to be multifactorial.

In conclusion, the microarray data presented illustrate cellular perturbations involving multiple signaling pathways and cascades as a possible consequence of systemic abnormal galactosylation of numerous cellular glycoproteins with possible abnormalities of protein glycan site occupancy, folding and survival, and the synthesis of glycolipids with incongruent genotype-phenotypes and outcomes in one pair of siblings, as we have described previously (4). We propose that the persistent abnormal glycosylation of serum glycoproteins observed in these treated patients with these microarray data gives an indication of the systemic malfunctioning and metabolic dyshomeostasis persisting in "treated" GAL, of a variable nature indicating that GAL does not function as a single gene disorder (33). Follow-up analyses will be required initially to determine whether any of the dysregulated pathways may be modified by relaxation of severe galactose restriction which could potentially improve galactose substrate for UDP-Gal and for galactosyl ceramides in these patients.

The restriction of dietary galactose is clearly life saving in the neonatal period. However, further studies are warranted to investigate the influence of modifier genes and accessory salvage pathways of galactose metabolism and individualized galactose tolerance (34). Follow-up studies are also needed to determine how inappropriate glycosylation impacts glycoprotein functioning in both adults and children with this systemic disorder.

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