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Hypoglycosylation with increased fucosylation and branching of serum transferrin *N*-glycans in untreated galactosemia

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Untreated classic galactosemia (galactose-1-phosphate uridyltransferase [GALT] deficiency) is known as a secondary congenital disorders of glycosylation (CDG) characterized by galactose deficiency of glycoproteins and glycolipids (processing defect or CDG-II). The mechanism of this undergalactosylation has not been established. Here we show that in untreated galactosemia, there is also a partial deficiency of whole glycans of serum transferrin associated with increased fucosylation and branching as seen in genetic glycosylation assembly defects (CDG-I). Thus galactosemia seems to be a secondary "dual" CDG causing a processing as well as an assembly N-glycosylation defect. We also demonstrated that in galactosemia patients, transferrin *N*-glycan biosynthesis is restored upon dietary treatment.

Key words: galactosemia/hyperfucosylation/hypoglycosylation/MALDI/transferrin

Introduction

Classic galactosemia results from the deficiency of galactose-1-phosphate uridyltransferase (GALT) (EC 2.7.7.12) in the Leloir pathway which is proposed to convert β-D-galactose to the more metabolically useful glucose-1-phosphate (Holden *et al.*, 2003). Initially galactokinase (EC 2.7.1.6) phosphorylates galactose to galactose-1-phosphate; this is converted to uridindiphosphate (UDP)-galactose through the action of GALT which transfers a uridine monophosphate (UMP) moiety from UDP-glucose to galactose-1-phosphate (Gal-1-P) thereby generating glucose-1-phosphate and UDP-galactose. In the last step of the Leloir pathway, UDP-glucose is regenerated from UDP-galactose by UDP galactose-4-epimerase (EC 5.1.3.2) and it interacts with another galactose-1-phosphate to go through this cycle again. In humans, defects of galactokinase, uridyltransferase, or epimerase give rise to inherited disorders referred to collectively as galactosemia (Novelli and Reichardt, 2000).

Classic galactosemia is a potentially lethal disease with clinical manifestations which usually begin in the neonatal period within a few days of milk ingestion. Untreated patients show failure to thrive, vomiting or diarrhea, cataracts, liver dysfunction, and episodes of severe hemolysis and intellectual impairment (Segal and Berry, 1995). The mechanisms producing dysfunction of these different organs are unknown yet; GALT deficiency leads to the accumulation of galactose-1-phosphate and to oxidation and reduction of galactose to galactonate and galactitol, respectively (Jacobs *et al.*, 1995; Wehrli *et al.*, 1997). Long-term complications in galactose-free diet patients include motor and verbal dyspraxia and premature ovarian failure. There is no correlation between outcome and genotype, residual GALT activity, or galactose-1-phosphate red blood cells (RBC) levels (Guerrero *et al.*, 2000; Robertson *et al.*, 2000).

In untreated galactosemic patients isoform patterns of serum transferrin, follicle stimulating hormone, and the lysosomal enzymes, β-hexosaminidase and α-fucosidase, are abnormal because of the increase of relatively neutral isoforms corresponding to less sialylated carbohydrate structures (Jaeken *et al.*, 1992; Prestoz *et al.*, 1997; Stibler *et al.*, 1997; Charlwood *et al.*, 1998). This is similar to that observed in the congenital disorders of glycosylation (CDG) which are genetic defects of glycan biosynthesis. CDG are classified as type I or II on the basis of the position of the defect in the glycosylation pathway. Type I consists of defects in the assembly or transfer of the dolichol-sugar intermediate, whereas type II refers to defects in the processing of the protein bound N-linked glycan (Grunewald *et al.*, 2002).

CDG type Ia (CDG-Ia) is due to phosphomannomutase (PMM) deficiency (Van Schaftingen and Jaeken, 1995), a key enzyme in the synthesis of guanosine 5′-diphosphate (GDP)-D-mannose which is required in the first steps of *N*-glycan biosynthesis. Clinically CDG-Ia and galactosemia share some clinical features including high frequency of death because of *Escherichia coli* sepsis in the neonatal period (Levy *et al.*, 1977), coagulopathy also with little evidence of liver disease (Levy *et al.*, 1996), hypergonadotropic hypogonadism, and intellectual impairment. It has been proposed that hypoglycosylation may be the key to some symptoms of galactosemia such as neurological deficits and hypogonadism through an action of Gal-1-P on the composition of membrane glycosphingolipids (gangliosides) of the developing central nervous systems and altered N-linked glycosylation pattern of secretory

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glycoproteins (Waggoner *et al*., 1990; Stibler *et al*., 1997; Segal, 1998; Lai *et al*., 2003). Subnormal glycosylation of glycolipids has been reported in galactosemia: lymphocytes and brain lipids of a galactosemic infant were deficient in galactosamine and galactosyl residues with respect to a nongalactosemic control (Petry *et al*., 1991).

Mass spectrometric methods, such as matrix-assisted laser desorption/ionisation (MALDI) and electrospray (ESI), owing to their growing technical improvements, have emerged as fundamental techniques in functional glycomics and glycoproteomics to establish a relationship between glycosylation changes and disease. Several protocols which allow rapid profiling and sequencing of *N*- and *O*-glycans have been developed on either underivatized or permethylated oligosaccharides (Harvey, 2001; Ciucanu and Costello, 2003; Mills *et al*., 2003; Spina *et al*., 2004). In this study we have analysed the glycosylation of intact serum transferrin and transferrin *N*-glycan structures from two untreated patients with galactosemia: notably, the patients had been both overexposed to dietary galactose (11 and 5 weeks) because of an initial false negative newborn screening following red blood cell transfusion. The results were compared to those observed in CDG-Ia patients and healthy individuals. In addition to transferrin underglycosylation, we have shown an increase in fucosylation and branching of transferrin *N*-glycans in the galactosemic patients which normalized upon dietary treatment.

Results

Analysis of human transferrin glycosylation

Human serum transferrin has two N-glycosylation sites at Asn^{413} and Asn^{611} which are normally occupied by biantennary complex-type structures terminating with α2,6-linked *N*-acetylneuraminic acid (NeuAc). The main fraction is diglycosylated transferrin (>95% according to Yamashita *et al*., 1993). A less amount of serum transferrin bears triantennary glycans, capped with α2,3-linked NeuAc on the β1,6-linked branch. A little fraction of these glycoforms is usually α1,6 fucosylated at the chitobiosyl core. These structures give rise to a quite homogeneous isoform pattern, so that alterations can be promptly revealed by changes in molecular weight and in pIs.

The isoelectric focusing (IEF) of serum proteins following transferrin immunodetection (Figure 1) revealed a severe underglycosylation in untreated galactosemic patients. The results obtained in untreated galactosemia were compared to those obtained in healthy controls and in subjects affected from CDG-Ia.

Transferrin IEF of normal human serum (Figure 1, lane a) showed a predominant band corresponding to the tetrasialo fraction while either in both the untreated galactosemic samples (lane b and lane c respectively for patient 1 and patient 2) and in CDG Ia (lane d), a cathodal shift generated, to different extents, additional bands in the positions of disialo and asialo isoforms, according to pIs changes due to the reduced glycosylation degree.

A fully automated online liquid chromatography (LC)–ESI method properly developed for the analysis of intact serum transferrin (Lacey *et al*., 2001) provided us a high resolution and sensitive diagnostic tool to individuate carbohydrate-deficient transferrins (CDT) in galactosemia. Although the deconvoluted ESI mass spectrum of rivanol-purified transferrin from normal subjects (Figure 2a) showed a unique molecular ion peak at 79,579.0 Da, corresponding to the diglycosylated species, in galactosemic patients the transferrin mass

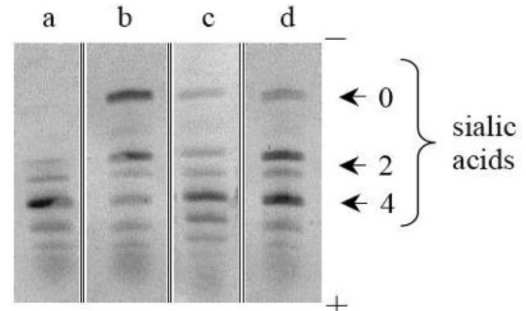

Fig. 1. Isoelectric focusing (IEF) pattern (pH 4.0–6.5) of serum transferrin: (**a**) normal control, (**b**) untreated galactosemic patient 1, (**c**) untreated galactosemic patient 2, and (**d**) and congenital disorders of glycosylation type Ia (CDG-Ia).

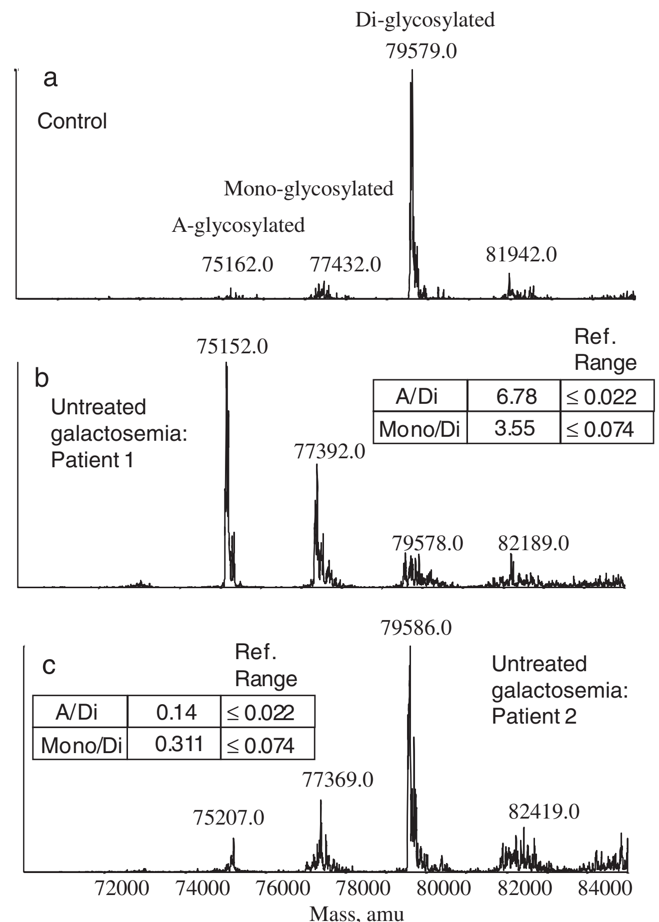

Fig. 2. Liquid chromatography–electrospray (LC–ESI) analyses of intact serum transferrins: (**a**) normal control, (**b**) untreated galactosemic patient 1, and (**c**) untreated galactosemic patient 2.

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spectrometric profiles underwent evident alterations because of additional peaks indicating the absence of one or both of the *N*-glycan moieties. In particular, patient 1 (Figure 2b) after a 11 weeks overexposure to dietary galactose showed a strong increase of the isoforms at 77,392.0 Da and 75,152.0 Da as demonstrated by the calculated abnormal glycosylation ratio ranges which were 3.55 (mono-/diglycosylation ratio) and 6.78 (a-/diglycosylation ratio), respectively. The mass spectrometry (MS) profile from patient 2 (Figure 2c), overexposed to dietary galactose for 5 weeks, similarly revealed the presence of CDT at 77,369.0 Da (mono-/diglycosylation = 0.311) and at 75,207.0 Da (a/diglycosylation = 0.14), also in this case over the reference ranges. These findings are in line with the lack of one or either the complete N-linked moieties, as seen in genetic glycosylation assembly disorders or CDG-I (Wada *et al.*, 1992), and indicate that defective glycosylation in galactosemia involves a substantial hypo-N-glycosylation of serum transferrin, which appears to reflect the exposure time to dietary galactose.

Characterization of N-linked glycans of human serum transferrin in untreated galactosemia

N-linked glycans were enzymatically released from rivanol-purified transferrin by peptide *N*-glycosidase F (PNGase F) and analysed by matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF MS), both in negative and in positive polarity.

A comparison of the mass spectra, acquired in negative ion polarity, of the acidic species derived from a healthy control and from the untreated galactosemic patients, respectively, is reported in Figures 3a-c and summarized in Table I which reports also the theoretical and the observed average masses, the corresponding predicted structures, and the relative percentage of the founded species. As expected, in normal transferrin (Figure 3a) the predominant species was the disialylated biantennary oligosaccharide at *m/z* 2222.8 (Table I, structure E). Further peaks were also identified as minor components and were assigned to biantennary and triantennary glycans, either unfucosylated and fucosylated at the core, and to the corresponding structures lacking one of the terminal sialic acids. These results are in agreement with the previously published glycan composition of normal transferrin (Dorland *et al.*, 1977; Spik *et al.*, 1985; Hoffmann *et al.*, 1995).

The analogous spectrum profiles related to galactosemic patients (Figure 3b and c) appeared much more heterogeneous as they showed a substantial increase in the number of peaks detected, attributable to mixtures of bi-, tri- and tetraantennary glycans. Also of note was the significant rise in the relative amount of the monofucosylated biantennary peaks at *m/z* 2077.9 and 2368.9 (species D and G reported in Table I), corresponding respectively to the mono and to the disialylated glycoform, and the identification of the disialyl difucosylated one at *m/z* 2514.7 (structure I). Further mono and difucosylated species were moreover detected at higher masses, like those associated to triantennary glycans at *m/z* 2733.9, 3025.2, and 3171.0 (species L, N, and O) and to tetraantennary structures at *m/z* 3391.5, 3682.7, and 3827.8 (named respectively P, R, and S). Table I moreover reports the qualitative and quantitative alterations, extrapolated from the MALDI mass spectra (data not shown), of the acidic glycans released from the whole serum. The results obtained were in full agreement with those observed for transferrin *N*-glycan profiling, as they showed for both patients a marked increment of fucosylated and branched species with respect to the reference control.

All these spectra were recorded in negative polarity according to Papac *et al.*'s procedure (1996) to avoid sialic acid fragmentation, but this approach leaves neutral species undetected. To identify the presence of truncated asialo glycoforms, we performed MALDI analysis of the same samples also in positive polarity. In this case *N*-glycans preferably desorbed as sodiated pseudomolecular ions $[M+Na]^+$. Figure 4a–c compares the corresponding mass spectra obtained from a control subject and from galactosemic patients. Even in positive ion mode this latter spectral profiles are more complex, especially in the low mass range between 1450 and 2200 Da, due to the appearance of ions corresponding to truncated biantennary species deficient in sialic acid and/or in galactose. In untreated galactosemia again fucosylated species were noted as peaks at about *m/z* 2392, 2101, and 1810 (corresponding to biantennary di-, mono-, and asialo glycoforms) as well as truncated undergalactosylated ions completely absent in the control.

Analysis of human serum transferrin in galactosemia after a galactose-free diet

As known (Gitzelman, 1995; Charlwood *et al.*, 1998), a galactose-free diet plays a fundamental role in lowering the level of Gal-1-P in RBC and contributes to a general improvement of the patient's state of health. From these observations, it was worthwhile to investigate the glycosylation state of transferrin and to perform the full characterization of its glycoforms after a prolonged dietary treatment (6 weeks long).

The high resolution ESI mass spectra of intact transferrin, obtained by rivanol treatment from serum of both patients under dietary restrictions (Figure 5b and c) showed a normal profile with amounts of aglycosylated and monoglycosylated forms within the standard reference ranges for pediatric patients. This represented a first clear sign of a restored normal *N*-glycan biosynthetic pathway.

Looking at the detailed transferrin isoforms we had the confirmations of the earlier hypothesis: the MALDI profiles either in positive and in negative polarity of the oligosaccharides deriving from treated galactosemic patients (data not shown) were very close to the corresponding obtained from normal subjects, as they contained mainly the typical biantennary disialylated moiety, with a very low content of species due to an abnormal glycosylation, and, above all, showing no evidence of increased fucosylation which had characterized the galactosemic samples.

Discussion

The results from this study indicate that the carbohydrate deficient serum transferrin obtained from the two long-term untreated galactosemic patients under investigation is mainly the result of the loss of the entire N-linked oligosaccharide at one or both N-glycosylation sites.

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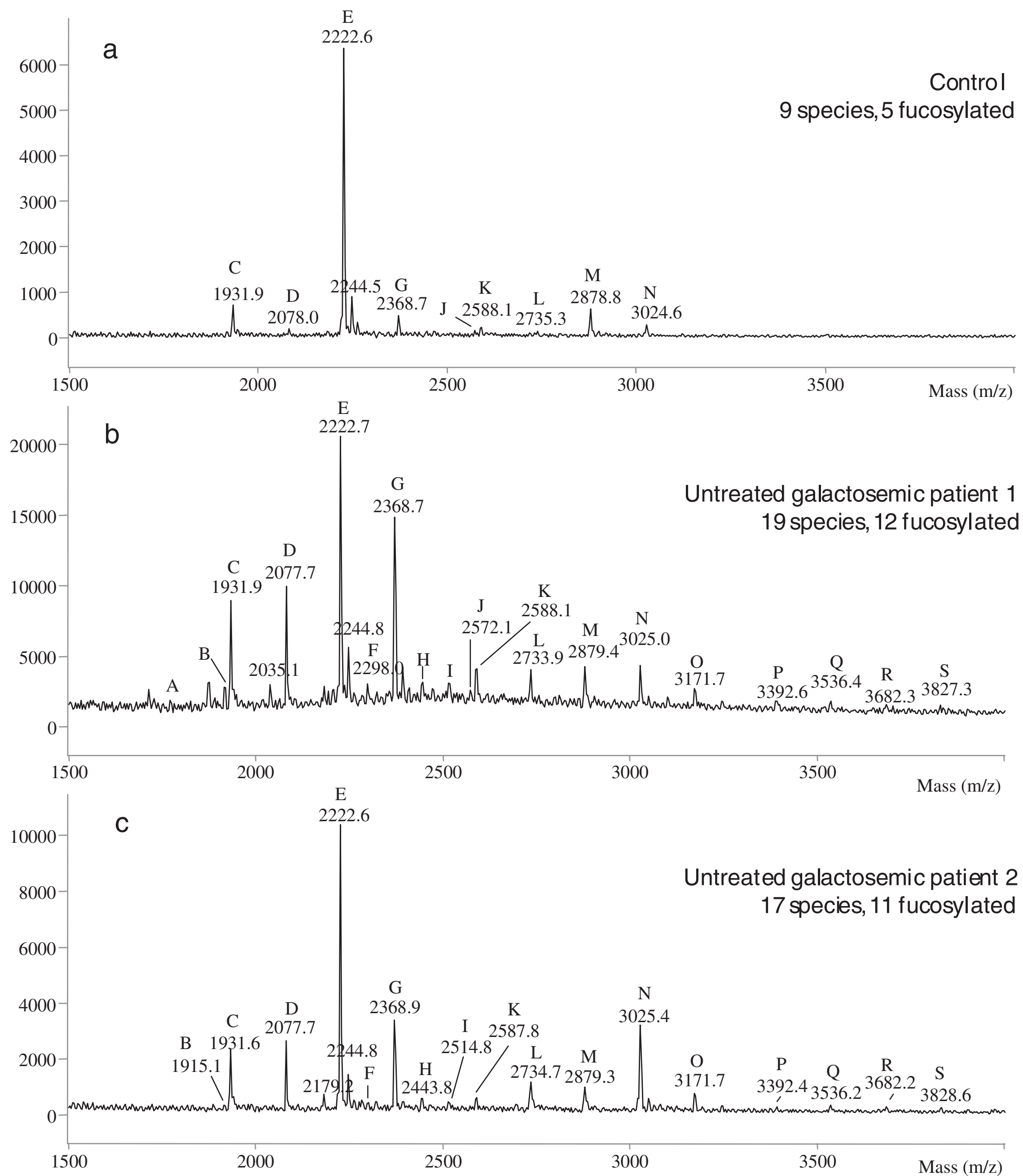

Fig. 3. Negative-ion (MALDI) spectra of the acidic *N*-glycans released from serum transferrin. (**a**) Healthy control, (**b**) untreated galactosemic patient 1, and (**c**) untreated galactosemic patient 2. Letters that have been used to represent the various structures are depicted in Table I.

In addition, we provide evidence for an increase in total fucosylation and branching of serum transferrin *N*-glycans, including bi-, tri-, and tetraantennary glycans, in patients with long-term untreated galactosemia (up to 19 sialylated species, 12 fucosylated) compared with the controls (9 species, 5 fucosylated). The specificity of liver α1,3 fucosyltransferase to transfer α1,3-linked fucose to α2,3 sialic acid terminating branches, to create the sialyl Lewis X structure, suggests that the increase in fucosylation of the biantennary glycans in transferrin is because of the core α1,6 fucosylation (Beyer *et al.*, 1979; Van Dijk *et al.*, 1995). However, the presence of the mono- and bifucosylated tri- and tetraantennary glycans indicates that either peripheral fucosylation of the antennae or core α1,6 fucosylation must occur in galactosemic patients.

A significantly increased degree of *N*-glycan core α1,6 fucosylation and peripheral α1,3 fucosylation has been previously reported for the whole serum *N*-glycome (Callewaert *et al.*, 2003), as well as for serum transferrin (Mills *et al.*, 2001), α-1-acid antitrypsin (Mills *et al.*, 2001), and α-1-acid glycoprotein (Van Dijk *et al.*, 2001) in all known types of CDG-I. On the contrary, no increment in the fucosylation levels of transferrin in CDG-II patients was recognized (Mills *et al.*, 2003); in this study, we moreover observed

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Table I. Structures of acidic *N*-glycans (referred to in Figure 3 and text) released from transferrin and total serum by purified transferrin by peptide *N*-glycosidase F (PNGase F) and identified by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS)

				Released % of *N*-glycans from rivanol isolated transferrin					Released % of *N*-glycans from total serum		
	Theoretical mass [M–H]⁻	Observed mass [M–H]⁻	Structure	Control	Untreated galactosemia patient 1	Untreated galactosemia patient 2	Treated galactosemia patient 1	Treated galactosemia patient 2	Control	Untreated galactosemia patient 1	Untreated galactosemia patient 2
A	1769.6	1769.6		–	7.5	–	–	–	–	14.2	6.8
B	1915.8	1915.3		Traces	10	3.2	3.0	–	Traces	Traces	6.0
C	1931.8	1931.8		9.4	34.2	19.8	35.3	11.1	22.6	30.0	35.1
D	2077.9	2077.9		2.5	38.0	22.5	6.3	5.9	11.9	33.1	24.8
E	2223.0	2222.8		100	100	100	100	100	100	100	100
F	2297.1	2296.6		–	12.2	Traces	–	–	–	12.0	Traces
G	2369.2	2368.9		6.5	72	28.8	10.6	8.6	10	55.6	36.9
H	2443.2	2443.5		–	12.3	5.1	–	–	8.6	12.3	6.7
I	2513.5	2514.7		–	12.9	4.0	–	–	–	10.2	5.1
J	2572.3	2572.1		2.1	9.8	Traces	3.9	–	–	Traces	4.7
K	2588.4	2588.1		3.1	15.7	5.3	7.4	5.2	8.7	11.5	7.3
L	2734.5	2733.9		1.8	24.0	10.0	4.3	2.7	8.1	11.7	13.7
M	2879.6	2879.2		9.9	24.5	11.8	9.6	8.9	12.0	20.3	14.7
N	3025.8	3025.2		3.6	24.7	32.2	5.6	7.4	15.4	20.8	24.8
O	3171.9	3171.0		–	10.2	7.3	Traces	–	–	8.2	9.1
P	3391.1	3391.5		–	6.9	2.7	–	–	–	7.1	3.7
Q	3536.2	3536.4		–	6.9	3.1	–	–	5.7	7.3	4.1
R	3682.4	3682.7		–	6.1	2.7	–	–	5.2	7.1	7.6
S	3828.5	3827.8		–	5.7	2.0	–	–	–	6.8	7.2
T	3974.7	3974.0		–	–	–	–	–	–	–	3.3

Black squares, *N*-acetylglucosamine; gray circles, mannose; white circles, galactose; diamonds, sialic acid; triangles, fucose.

(data not shown) that also in CDG-Ia patients fucosylation and branching of serum transferrin *N*-glycans are increased, although less pronounced, than that observed in galactosemic subjects. As a whole we demonstrate that in long-term untreated galactosemia as well as in CDG-I there is an underoccupancy of N-glycosylation sites and additional findings of increased fucosylation and branching of *N*-glycans. Therefore, it is possible that in both of these disorders, the underglycosylation of serum transferrin could result in a decreased flux of molecules through the Golgi, leading to a greater processing of individual glycoproteins.

In CDG-Ia patients, hyperfucosylation has been attributed to a chronic hepatic inflammatory state (Van Dijk *et al.*, 2001; Callewaert *et al.*, 2003). Moreover, the extent of

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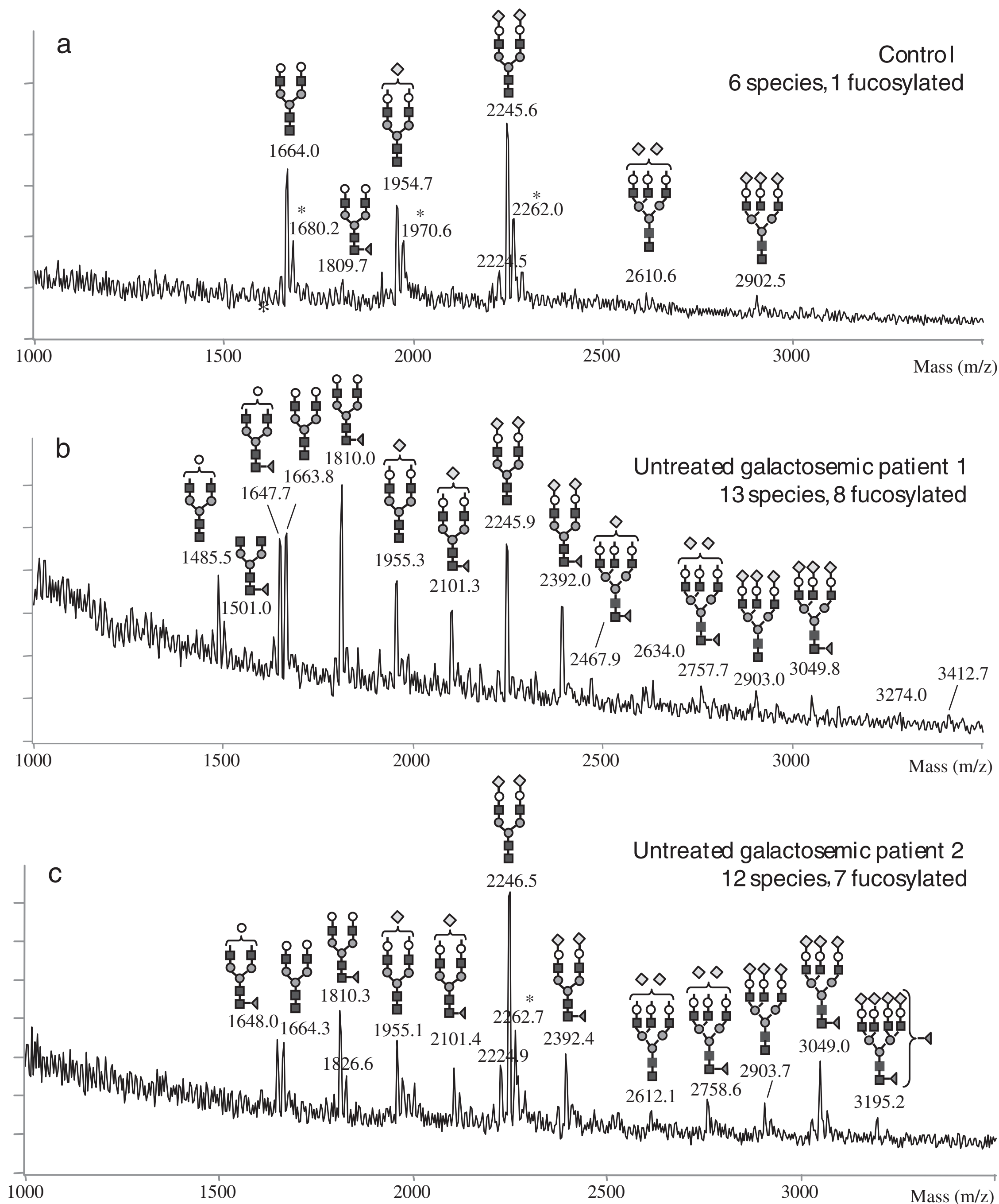

Fig. 4. Positive-ion matrix-assisted laser desorption/ionisation (MALDI) spectra of transferrin *N*-glycans from a (**a**) healthy control and (**b** and **c**) untreated galactosemic patients 1 and 2. Oligosaccharides desorb preferably as $[M+Na]^+$ species, whereas ions indicated with asterisks are $[M+K]^+$ adducts. Black squares, *N*-acetylglucosamine; gray circles, mannose; white circles, galactose; diamonds, sialic acid; triangles, fucose.

branching and fucosylation of serum glycoproteins is a finding of chronic inflammatory diseases where it correlates with the increase in the expression of processing enzymes and serum fucosyltransferase activity (Becker and Lowe, 2003; Callewaert *et al.*, 2003). Interestingly, both present patients with long-term untreated galactosemia showed clinical and laboratory changes consistent with a prolonged liver inflammatory state at the time of the study. It would be informative to assess the degree of transferrin N-glycan fucosylation and the extent of branching in galactosemic patients with respect to the rate and the extent of exposure to dietary galactose.

The intracellular reduction of essential uridylated hexoses in galactosemic patients might inhibit protein glycosylation (Lai *et al.*, 2003) and contribute to this findings of serum transferrin underglycosylation; notably, after treatment the proportion of the highly branched glycans and hyper-fucosylated derivates decreased and the proportion of the

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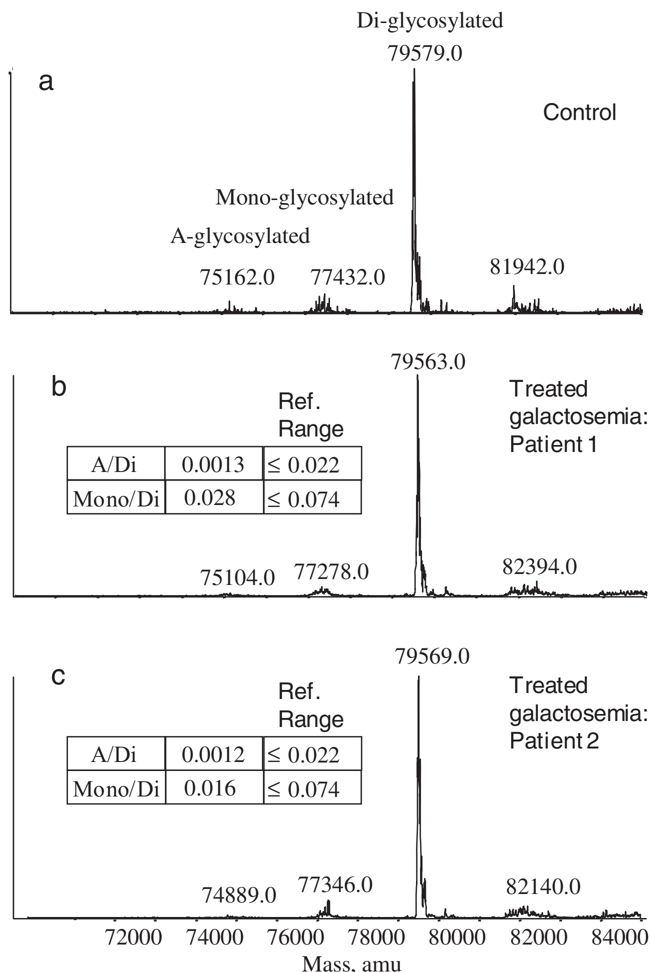

Fig. 5. Liquid chromatography–electrospray (LC–ESI) analyses of intact serum transferrins: (**a**) normal control, (**b**) treated galactosemic patient 1, and (**c**) treated galactosemic patient 2.

disialylated biantennary glycan increased, reflecting normalization of the glycosylation. Classic galactosemia has been classified as a secondary CDG (Jaeken and Carchon, 1993). These observations point to galactosemia as a secondary "dual" CDG with an assembly defect as well as a processing defect (secondary CDG-I/II).

Materials and methods

Patients

The two male unrelated galactosemia patients were Italian.

Patient 1. This galactosemia infant was misdiagnosed after an initial positive screening test result because he received packed red cell transfusion in the first few weeks of life. At this time, he was considered to have GALT activity in the heterozygous range (3.3 U/g Hb; normal levels: 14–25; heterozygous levels: 0.5–13.9). As he was clinically symptomatic with icterus, poor feeding and weight loss, dietary galactose restriction was temporary performed between weeks 3 and 20, with clinical amelioration. Then, he was fed with cow milk between weeks 21 and 32. We first observed him at 32 weeks because of weight loss, vomiting, and easy irritability. Abnormal laboratory tests included Hb 9.9 g/dL (nv 11–13), SGOT 204 IU/L, SGPT 217 IU/L (nv 0–50), blood protein levels 4.8 g/dL (nv 5.5–6.5). GALT activity in RBC was absent and RBC galactose-1-phosphate level was 8.2 mg/dL (nv < 0.3). Molecular analysis for GALT mutations was not performed.

Samples of patient serum for transferrin glycosylation analyses and characterization of *N*-glycan structures were obtained following 11 weeks overexposure to dietary galactose intake (32 weeks) and 6 weeks after the introduction of a galactose-free diet.

Patient 2. The infant presented with vomiting and failure to thrive after a few days of milk ingestion. He received packed red cell transfusion because of severe hemolysis at 3 weeks and he was started on galactose-free diet at week 5. At this time, pertinent laboratory findings included increased serum transaminases levels (SGOT 198 IU/L, SGPT 205 IU/L, nv 0–50); GALT activity in RBC was 10.9 μmol/g (Hb) per hour and Gal-1-P 7.4 mg/dL. On week 12 GALT activity in RBC was 0.3 μmol/g (Hb) and Gal-1-P was 4.1 mg/dL. Mutation analyses of GALT gene by sequencing exon 1–11 showed a frameshift mutation T6fsdelC on exon 1 and R333W mutation on exon 10.

Samples of patient serum for transferrin glycosylation analyses and characterization of *N*-glycan structures were obtained at 6 weeks, that is after 5 weeks overexposure to dietary galactose and 6 weeks from the introduction of dietary galactose restriction.

Patient samples

Serum from galactosemic subjects was collected before an after treatment as described in galactosemic patients background. Control serum was obtained from age-matched volunteers and CDG-Ia patients. Informed consent was obtained for all sera samples.

Purification of transferrin

Human transferrin was obtained from aliquots (200 μL) of serum by rivanol treatment and two subsequent precipitations with NaCl 25% (w/v) and saturated ammonium sulfate (Charlwood *et al.*, 1998). The supernatant was purified and transferred at the same time to a 20 mM phosphate buffer pH 7.5 on a CentriPor centrifuge concentrator MWCO 25,000 (Spectrum Microgon, Houston, TX), before the quantitative protein test by Lowry assay (Lowry *et al.*, 1951).

Release and purification of transferrin N-linked glycans

Transferrin (100 μg) was incubated overnight at 37°C with 100 U (3 μL) of PNGase F (Roche Diagnostics).

The released oligosaccharides were purified by solid-phase extraction on GlycoClean H graphite cartridges (Prozyme, San Leandro, CA).

Release and purification of total serum glycoproteins N-linked glycans

Five to ten microliters of serum from both galactosemic patient (either untreated and before a six weeks galactose-free diet)

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and from three control subjects were incubated at 50°C for 1 h with 50 μL of RCM buffer (8 M urea, 360 mM Tris, pH 8.6, 3.2 mM ethylenediaminetetraacetic acid [EDTA]). The denaturated serum proteins were afterwards loaded on a 96-well MultiScreen assay system (Millipore, Billerica, MA) equipped with a MultiScreen-IP plate (pore size 0.45 μm, Millipore) following the enzymatic deglycosylation procedure described by Papac *et al.* (1998). Also in this case the released *N*-glycans were purified by solid-phase extraction on GlycoClean H graphite cartridges (Prozyme).

Sample preparation and MALDI-TOF analysis

The oligosaccharide mixtures containing above all sialylated species were first converted in the ammonium form by a home-made miniaturized column of cation-exchange resin Dowex 50WX8-200 (Sigma-Aldrich, Basel, Switzerland) previously equilibrated in a 5% NH_4OH solution. Glycans were eluted with water and dried in a centrifugal concentrator (SpeedVac Thermo Savant, Holbrook, NY), then were dissolved in a few microliters of 0.1% trifluoroacetic acid (TFA) before MALDI analyses. The obtained samples were analysed either in negative polarity, by using 2′,4′,6′-trihydroxyacetophenone (THAP) in acetonitrile/20 mM ammonium citrate (50/50 v/v) as matrix solution (Papac *et al.*, 1996), and in positive polarity in 2,5-dihydroxybenzoic acid (DHB) 50 mg/mL TFA 0.1% acetonitrile 80/20. Recrystallization from methanol was performed according to Spina *et al.*'s procedure (2000).

Mass spectra were acquired in linear mode on a voyager STR instrument (Applied Biosystems, Framingham, MA) equipped with a nitrogen laser (λ 337 nm) and provided with delayed extraction technology. Ions formed by the pulsed laser beam were accelerated through 24 kV.

ESI–MS

ESI–MS experiments were performed as described by Lacey *et al.* (2001). Briefly, an API 3000 triple quadrupole mass spectrometer (Perkin–Elmer Sciex, Toronto, Ontario, Canada) operated in ion evaporation mode with the TurboIonSpray ionization probe source (operated at 5500 V) was used. Peripherals included two Perkin–Elmer Series 200 micropumps, Perkin–Elmer Series 200 autosampler (Norwalk, CT), and a Shimadzu system controller, SCL-10Avp, which controls two Shimadzu liquid chromatography LC 10 ADvp pumps (Columbia, MA) and two valves (Valco Instruments).

Human transferrin was eluted from the C4 column and introduced to the TurboIonSpray source using 0.5% acetic acid–0.02% trifluroacetic acid/methanol/acetonitrile (5/48/48) at a flow rate of 50 mL/min. The TurboIonSpray source was operated with turbo gas on (6 L/min; sensor temperature at 150°C) with the effluent flow splitting at 1:2. The MS was operated in Q1 scan mode from 2000 to 3000 amu with a transferrin retention time of 7 min and complete analysis time of 9 min. Total instrument acquisition time was 9.5 min per sample that includes all steps previously described. Data were acquired and processed using the Mass-Chrom software (version 1.1.2, Perkin–Elmer Sciex) including BioMultiView, version 1.3.1. The BioSpec–Reconstruct algorithm was used to deconvolute charge distribution raw data to reconstructed mass data. Specifically, multiply charged spectra were transformed through five iterations using input data between *m/z* 2000 and 3000 and a transformed output data range of 74,000–81,000 Da.

IEF

IEF of serum transferrin was performed as described by Stibler *et al.* (1991).

Serum samples, saturated with ferric citrate (40 μM) in the presence of sodium bicarbonate (50 mM), were run on agarose gels, in a pH range of 4.0–6.5, on a Phast-System (Amersham Biosciences, Uppsala, Sweden). The focused transferrin isoforms were fixed by immunoprecipitation with a goat anti-human transferrin IgG (Sigma Chemical, St. Louis, MO). The unbounded proteins were washed out and the gels were stained with Coomassie blue R250.

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Abbreviations

CDG, congenital disorders of glycosylation; CDT, carbohydrate-deficient transferrin; DHB, 2,5-dihydroxybenzoic acid; ESI, electrospray, Gal-1-P, galactose-1-phosphate; GALT, galactose-1-phosphate uridyltransferase; IEF, isoelectric focusing; MALDI-TOF MS, matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry; NeuAc, *N*-acetylneuraminic acid; PNGase F, protein *N*-glycanase F; RBC, red blood cells; TFA, trifluoroacetic acid; UDP, uridindiphosphate.

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