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Screening for abnormal glycosylation in a cohort of adult liver disease patients

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Abstract

Congenital Disorders of Glycosylation (CDG) are a rapidly expanding group of rare genetic defects in glycosylation. In a novel CDG subgroup of Vacuolar-ATPase assembly defects various degrees of hepatic injury have been described, including end stage liver disease. However, the CDG diagnostic workflow can be complex as liver disease per se may be associated with abnormal glycosylation. Therefore, we collected serum samples of patients with a wide range of liver pathology to study the performance and yield of two CDG screening methods. Our aim was to identify glycosylation patterns that could help to differentiate between primary and secondary glycosylation defects in liver disease.

To this end, we analyzed serum samples of 1042 adult liver disease patients. This cohort consisted of 567 liver transplant candidates and 475 chronic liver disease patients. Our workflow consisted of screening for abnormal glycosylation by transferrin isoelectric focusing (tIEF), followed by in-depth analysis of the abnormal samples with quadruple time-of-flight mass spectrometry (QTOF-MS).

Screening with tIEF resulted in identification of 247 (26%) abnormal samples. QTOF-MS analysis of 110 of those did not reveal glycosylation abnormalities comparable with those seen in V-ATPase assembly factor deficiencies. However, two patients presented with isolated sialylation deficiency. Fucosylation was significantly increased in liver transplant candidates compared to healthy controls and patients with chronic liver disease.

In conclusion, a significant percentage of patients with liver disease presented with abnormal CDG screening results, however, not indicative for a V-ATPase assembly factor defect. Advanced glycoanalytical techniques assist in the dissection of secondary and primary glycosylation defects.

Keywords N-glycosylation, hyperfucosylation, end-stage liver disease, V-ATPase assembly factor deficiencies, Congenital Disorders of Glycosylation

Take home message

Secondary glycosylation defects are common in liver disease and advanced glycoanalytics are needed to differentiate these from primary glycosylation defects such as the V-ATPase assembly factor deficiencies.

Compliance with Ethics Guidelines

Conflict of Interest

Jos C. Jansen, Bart van Hoek, Herold J. Metselaar, Aad P. van den Berg, Fokje Zijlstra, Karin Huijben, Monique van Scherpenzeel, Joost P.H. Drenth and Dirk J. Lefeber all declare they have no conflict of interest.

Informed Consent

Approval was granted by the Ethics Committee of the Radboudumc and documented in case file 2018-5012. All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2000.

Animal Rights

This article does not contain any studies with human or animal subjects performed by any of the authors.

Details of the contributions of individual authors

JJ, MvS, JD and DL design the study. JJ, FZ and KH performed the experiments. BvH, HM, AvdB and JD provided samples and collected data. JCJ, JD and DL wrote the manuscript. All authors critically reviewed the manuscript.

1. Introduction

Congenital disorders of glycosylation (CDG) are a group of inborn errors of metabolism characterized by abnormal glycosylation. Most CDG share a multisystem phenotype with dysmorphic features, failure to thrive and neurological symptoms.(Peanne et al 2017) Involvement of the liver is frequent in CDG but usually not the dominant feature. (Marques-da-Silva et al 2017)

In recent years, a novel subgroup of CDG patients has emerged that presents predominantly with a hepatic phenotype. (Jansen et al 2016; Jansen et al 2016; Jansen et al 2016; Rujano et al 2017; Cannata Serio et al 2020) Pathological variants in this group are in genes that code for assembly factors of the vacuolar -ATPase (V-ATPase), the proton pump for intracellular acidification. The glycosylation pattern resembles a type 2 CDG with loss of sialic acid and galactose. The hepatic clinical spectrum ranges from mildly elevated serum transaminases and steatosis, resembling non-alcoholic fatty liver disease, to cirrhosis and end-stage liver disease warranting liver transplantation (LTx).

Liver cirrhosis develops as a response to chronic liver injury. The central pathological event in cirrhosis is deposition of extracellular matrix increasing hepatic flow resistance with ensuing hepatocyte dysfunction.(Tsochatzis et al 2014) Patients with cirrhosis have a high risk of decompensation of their liver disease. It can develop in end-stage liver disease (ESLD), necessitating (LTx)

Abnormal glycosylation is a known phenomenon in chronic liver disease and can be used to discriminate between different fibrosis stages and cirrhosis and provides an interesting and non-invasive alternative for liver biopsy.(Callewaert et al 2004). Abnormal glycan structures of liver-derived proteins such as transferrin (TF) and haptoglobin have been described in alcoholic liver

disease, non-alcoholic steatohepatitis and primary sclerosing cholangitis.(Blomme et al 2009; Clarke et al 2017; Culver et al 2018) These abnormalities include hyperfucosylation due to increased fucosyltransferase activity and hyposialylation as a result of lower sialyltransferase activity.

Traditionally, new CDG patients are identified through isoelectric focusing of TF (tIEF).(Francisco et al 2019) TF possesses two biantennary glycans at amino acids Asn432 and Asn630, both negatively charged because of the terminal sialic acids.(Guillard et al 2011) tIEF uses loss of these sialic acids to separate the various isoforms. A disadvantage of tIEF is that it only provides information on desialylation and not for example on hypogalactosylation and fucosylation. The use of quadruple time-of-flight mass-spectrometry (QTOF-MS) may overcome this disadvantage by providing in-depth high resolution information on the glycans attached to TF.(van Scherpenzeel et al 2015)

The primary aim of this study was to identify glycosylation patterns that could help to differentiate between primary and secondary glycosylation defects in liver disease within a cohort of adult patients with end-stage liver disease. As a secondary aim, we studied to what extent liver disease affects CDG screening.

2. Materials and methods

2.1 Selection of liver disease patients, sample selection and ethical considerations

We collected 1042 samples from patients with an established chronic liver disease from four Dutch tertiary referral hospitals. (Figure 2) Samples were collected in the period 1993 to 2013. We specifically targeted ESLD patients who were evaluated and waitlisted for LTx. These samples were

provided by Erasmus MC in Rotterdam (n= 264), LUMC in Leiden (n=142) and UMCG in Groningen (n=155). All samples were drawn and aliquoted preceding LTx.

Chronic liver disease samples (n=410), without ESLD, were provided by the Radboudumc in Nijmegen and by the LUMC (n=65, all with a diagnosis of auto-immune hepatitis). These patients were seen at the outpatient clinic for diagnosis and treatment of a range of liver diseases. Patients with viral hepatitis (infectious hepatitis B, C or E) were excluded from analysis as we hypothesized that the presence of underlying CDG would be unlikely in this patient population. Samples from healthy controls (n=40) were obtained from the local bloodbank. All samples were stored at -80 °C till analysis.

Material was collected in agreement with the Dutch code of conduct for responsible use of human tissue (Dutch Federation of Biomedical Scientific Societies, www.federa.org). All experiments were performed in accordance with the guidelines and regulations of the Ethics Committee of the Radboudumc. Approval was documented in case file 2018-5012.

2.2 Study design and workflow

We first performed tIEF of the collected samples to identify global N-glycosylation defects with hyposialylation. Sixty-one samples were excluded prior to tIEF: 24 samples because they were drawn after liver transplantation, 21 were of very poor quality and unsuitable for further workup, 5 samples were wrongly allocated, 10 patients were <18 years of age at time of sampling and one sample was from a patient with an established diagnosis of CDG.

We defined abnormal TF sialylation as an increased percentage of hyposialylated TF isoforms compared with the main isoform, tetrasialo TF. Control ranges were used from the clinical diagnostic

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protocol, derived by tIEF of 59 healthy control samples: asialoTF 0-3.2%; monosialoTF 0-5.0%; disialoTF 3.3-7.6%; trisialoTF 4.9-10.6%; pentasialoTF 18.7-31.5%.

If applicable, we designated the profile type 1 or type 2 CDG based on international consensus. Type 1 CDG has increased asialo- and disialo TF isoforms, indicating loss of 1 or 2 glycans. Type 2 CDG has hyposialylation for all TF isoforms.(Lefeber et al 2011)

Abnormal tIEF samples were selected for further work-up with QTOF-MS. Quality criteria for inclusion of the sample included an abundance of >50.000 amu of the intact TF glycoprotein (79556 amu). We selected peaks corresponding to a known TF isoform with an abundance of >1000. The relative abundance of these peaks was calculated based on their percentage relative to total abundance. Figure 1 shows a typical tIEF and QTOF-MS pattern and depicts the nomenclature of the glycosylation isoforms used throughout this manuscript.

2.3 Sample preparation for N-glycan analysis

Transferrin isoelectric focusing

All samples were analyzed with tIEF to determine abnormal sialylation of TF. tIEF was performed as described before. (Guillard et al 2011) Briefly, serum or plasma samples were incubated with iron and applied to a 5-7 pH gradient gel for electrophoresis. After completion, gels were incubated with 2.5 µl/cm² anti-TF antibody (Dako #A0061, Carpinteria, CA USA) and visualized with Coomassie blue. Data analysis was performed with Image Quant Software (Totallab, Newcastle upon Tyne, UK)

TF polymorphisms were recognized by doubled bands for all isoforms. For confirmation, neuraminidase treatment was applied on samples with ambiguous isoelectric patterns. (Guillard et al 2011)

Nano liquid chromatography-chip (C8)- quadruple time of flight mass spectrometry (QTOF-MS)

Mass spectrometry analysis was performed as described before.(van Scherpenzeel et al 2015) First, beads were loaded with anti-TF antibody (Dako #A0061, Carpinteria, CA USA) and stored in 20% ethanol. Prior to usage, beads were washed 4 times with a Tris-HCl (pH 7) solution. Next, 100 µl of a 1:10 plasma sample dilution in 0.9% sodiumchloride was mixed with beads and incubated for 20 minutes under continuous shaking at 3000 rpm/min. Subsequently beads were washed four times with Tris-HCl (pH 7) solution. For elution, 1 µl of Tris-HCl pH 9 solution was added to the sample followed by 50 µl elutionbuffer (0.1M Glycine-HCl pH 2.7). After spinning and verification of neutral pH, 2µl sample was injected into the microfluidic 6540 HPLC-chip-QTOF instrument (Agilent Technologies, Santa Clara, CA, USA). For data analysis of QTOF-MS profiles Agilent Mass Hunter Qualitative Analysis software (v. B.04.00) was used.

2.4 Statistical analysis

For statistical analysis SPSS Statistics v. 22 (IBM Corporation, Armonk, NY, USA) was used. Due to non-linearity of our data we used the non-parametric Kruskall-Wallis test for comparisons of more than two groups and the Mann-Whitney U test when there were two groups. P-values were adjusted for multiple testing with the Bonferroni method.

3. Results

A total of 1042 samples were collected, 567 samples of LTx candidates and 475 samples of patients with CLD. Figure 2 shows a flowchart of the study design. We included 981 samples for further N-

glycan analysis with tIEF. After tIEF, 744 samples including 20 samples with a confirmed polymorphisms were excluded based on a normal profile

Subsequently, QTOF-MS was performed on 247 samples and 40 healthy controls. After QTOF-MS analysis, 125 samples were excluded because the main peak (at 79556 amu) was of insufficient abundance, 10 samples were excluded because of an abnormal glycation profile and 3 because of a TF polymorphism (of which 2 were not identified with tIEF and one was a healthy control). In total, 110 patient samples and 39 healthy control samples were of sufficient quality for interpretation of the glycosylation profile. Table 1 shows patient characteristics for the selected samples.

Supplementary table 1 shows tIEF and QTOF-MS results for all samples with an abnormal tIEF profile.

3.1 tIEF screening of 961 samples of liver disease patients shows mild glycosylation abnormalities

Out of 961 samples we analyzed using tIEF, 247 samples (26%) had hyposialylation compared to our controls. Of these samples, 175 (70%) had a solitary increased percentage of the trisialoTF isoform, 42 (17%) had an elevated percentage of monosialoTF isoform and 4 (2%) had an elevated percentage of the disialoTF isoform. Mixed combinations of elevated isoforms occurred in 26 (11%) samples. (Figure 3a) None of the samples had increased percentages of all isoforms. One sample had slightly increased percentages of the asialo and the disialoTF isoform, and did not reach the values to be suggestive for type 1 CDG. The mixed profiles can be compatible with a CDG type 2 profile, however, in most samples the increase in percentage is subtle (Figure 3b).

3.2 QTOF-MS analysis of preselected liver disease patients did not identify profiles compatible with V-ATPase assembly factor defects.

Of the 110 high quality QTOF-MS profiles, none had a distinguishable type 1 CDG pattern. The peak associated with non-glycosylated TF (peak 75140) was only present in one LTx patient with progressive familial intrahepatic cholestasis syndrome (1.2% of total glycan abundance), one CLD patient with auto-immune hepatitis (1.1%) and three HC (all <1% total glycan abundance). Both patients had a normal percentage of asialoTF in tIEF screening and the six patients with an elevated asialoTF fraction with tIEF analysis did not have a detectable peak with mass 75140, corresponding to non-glycosylated transferrin. Peak 77350, which corresponds to loss of one glycan, was present in all but five samples and was not significantly different among the groups (data not shown). We did not identify samples that showed typical glycan structural abnormalities that are seen in type 2 CDG profiles and did not identify a pattern compatible with a V-ATPase assembly factor deficiency.

We identified two samples ($2/110 = 1.8\%$) with clearly elevated trisialo TF isoform abundance. The first patient in the CLD group was a female, age 55 at sampling, who was seen at the outpatient clinic for hepatic steatosis. Her profile showed an elevated trisialo TF isoform of 15.0%, (median for the CLD group is 2.0%) and presence of an additional isoform (mass 78976, corresponding with loss of 2 sialic acids). There were no signs of other glycosylation abnormalities. The second patient was a male liver transplant recipient diagnosed with alpha-1 anti-trypsin deficiency. At age of sampling he was 28 years old. His QTOF-MS profile showed an increase in the trisialo TF isoform of 12.4% (median for the LTx group is 1.9%) and also an increased loss of 2 sialic acids (mass 78976). For both patients, we could not detect transferrin glycoforms with missing galactose residues, as for example seen in the V-ATPase assembly defects.

3.3 tIEF but not QTOF-MS shows that loss of one sialic acid is more frequent and more severe in LTx candidates compared to CLD patients and HC

To see what the effect is of liver disease on desialylation we compared the tIEF screening results between LTx and CLD samples. Table 2 shows the medians for all isoforms. The prevalence of several isoforms were statistically significantly different between these groups, but medians where within the normal range. Only the LTx group had a median of the trisialo TF isoform above the upper limit of normal and significantly higher than in the chronic liver disease group (11.8% vs 10.4%, p <0.001). An abnormal tIEF pattern was more frequently seen in LTx candidates compared to CLD patients (175/511=34% vs. 72/450=16%, Chi square p-value =0.000).

Next, we aimed to gain more insight in desialylation with QTOF-MS. QTOF-MS provides additional detail on glycan composition. To investigate desialylation we used the combined abundance of the trisialo and the fucosylated trisialo TF isoform. Comparison of these combined peaks among the three groups only showed a slight statistically significant difference between the LTx and CLD groups (Supp. Table 2). However, because of the broad standard deviations we conclude that overall desialylation is not different between LTx candidates, CLD patients and healthy controls. In conclusion, based on these data, desialylation is more prominent in LTx candidates when measured with tIEF, but this data is not reproduced with QTOF-MS.

3.4 Hyperfucosylation more pronounced in end-stage liver disease than in chronic liver disease

Hyperfucosylation of liver derived proteins is a known phenomenon in a variety of liver diseases. Therefore, we calculated the fucosylation ratio for the trisialo and pentasialo TF isoforms. (Figure 4 and Supp. Table 2). Fucosylation of the tetrasialo TF isoform was not reliably detectable because of overlap with other nearby peaks. Figure 4 shows that the ratios are higher for the LTx samples compared to the HC and the CLD samples (p<0.0001 for both). These data indicate that

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hyperfucosylation of TF is more pronounced in end-stage liver disease compared to chronic liver disease and healthy controls.

4. Discussion

In our retrospective analysis of a cohort of 1042 liver disease patients we found a significant percentage of patients with altered CDG screening results by serum transferrin isoelectricfocusing. However, we did not find clear evidence for the presence of a CDG due to V-ATPase assembly factor defects. We found that hyperfucosylation is more pronounced in LTx patients compared to those with milder liver disease and healthy controls, thereby showing that high-resolution mass spectrometry aids the discrimination between secondary and primary glycosylation abnormalities in CDG screening.

4.1 Screening for possible primary glycosylation defects

Initial screening with tIEF resulted in identification of 247 (26%) , mostly mild, abnormal patterns. We did not identify a typical CDG type 1 pattern, which we anticipated, as to date there are no known type 1 CDG associated with a mild hepatic phenotype.(Peanne et al 2017) The majority of samples had an increase in the trisialo TF isoform. None had an increase of all isoforms, a feature of most type 2 CDG. However, based on these tIEF results we could not rule out a V-ATPase assembly factor deficiency as mild abnormalities have been described. QTOF-MS analysis did not confirm desialylation as seen in tIEF and did not show patterns compatible with type 1 CDG or V-ATPase assembly factor deficiencies. Similarly, no samples with loss of galactose were observed. Therefore

we conclude that our analysis did not identify novel patients with a V-ATPase assembly factor defect.

A possible reason for this might be that prevalence is too low for detection within our cohort. The exact prevalence of the whole group of CDG is unknown, but is estimated at 0.1 – 0.5 / 100.000 in Europe. This number is based on recent data from the European CDG network that estimated that there is a total number of 2500 recognized CDG patients in Europe. (Peanne et al 2017) However, these observations are based on patients with a multisystem phenotype. The discovery of the V-ATPase-CDG subgroup with a very mild hepatic phenotype could indicate that the prevalence is much higher. We speculated that this subgroup would be enriched in a population that covers the full spectrum of liver disease severity, but no cases were detected.

Another reason might be that glycosylation defects of CDG patients who survive into adulthood can become milder, or even normalize, over time. (Westphal et al 2001; Ng et al 2013; Wolthuis et al 2014). Spontaneous normalization of the glycosylation profile has not yet been investigated for V-ATPase-CDG. However, spontaneous normalization of serum transaminases has been described with this condition. (Jansen et al 2016)

We identified two patients with an abnormally high percentage of the isoform with loss of one sialic acid. The reason for this abnormal pattern remains unknown. Glycosylation defects in SLC35A1-CDG (OMIM 603585), a defect in the CMP-sialic acid transporter, show pure desialylation effects (Martinez-Duncker et al 2005; van Scherpenzeel et al 2015). However, the phenotype of SLC35A1-CDG is mostly neurological with dysmorphic features, not hepatic.

Also, the presence of bacterial sialidase in serum can lead to hyposialylation of TF. This has been described for *Streptococcus pneumoniae*- associated hemolytic ureum syndrome.(Scobell et al 2019). However, hemolytic uremic syndrome is primarily a disease of infancy and early childhood and the incidence in the adult population is extremely low.

Another option is that these patients have an as yet unrecognized type of CDG. Our study design did not allow us to further investigate this as we did not have access to fresh plasma, fibroblasts or parental DNA to perform adequate genetic analyses and functional studies.

4.2 Glycosylation defects in liver disease

Desialylation has been mostly studied in the context of alcoholic liver disease. Indeed, the Carbohydrate Deficient Transferrin (CDT) test to identify chronic alcohol intake is based on desialylation of transferrin. Analysis of CDT in abstaining patients with various degrees of liver disease shows a correlation of high CDT percentages with the Child-Pugh score.(DiMartini et al 2001)

The pathophysiological mechanism behind alcohol-induced hypoglycosylation is not fully elucidated. Some studies suggest a primary ER defect (Cottalasso et al 2002; Flahaut et al 2003). Other studies suggest an effect on the Golgi apparatus.(Ghosh et al 1995; Gong et al 2007). One study investigated gene expression of glycosylation genes in NASH, but found mixed results with upregulation of *ST6GAL2* and downregulation of *ST6GAL1*. (Clarke et al 2017)

Hyperfucosylation of liver derived proteins has been most extensively studied in hepatocellular carcinoma patients. (Pompach et al 2013; Huang et al 2017; Zhang et al 2017; Verhelst et al 2020) Indeed, fucosylated alpha-fetoprotein is an established disease marker for HCC in the

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setting of cirrhosis.(Sato et al 1993) Here we show that fucosylation of transferrin is elevated in samples from LTx candidates compared to CLD patients and healthy controls.

Hyperfucosylation of transferrin was shown to be the cause of chromatographic abnormalities in CDT testing, or so-called “di-tri-bridging” (i.e. poor resolution of disialoTF from trisialoTF).(Landberg et al 2012) Di-tri-bridging is associated with liver disease and is more frequent in cirrhosis than in non-cirrhotics.(Stewart et al 2017)

Somewhat older data exists on fucosylation of haptoglobin in liver disease and shows hyperfucosylation in patients with alcoholic liver disease and primary biliary cholangitis.(Mann et al 1994) A more recent paper showed increased hyperfucosylated glycans on haptoglobin in HCC as well as in cirrhosis. (Zhu et al 2014)

Previous work on serum glycan analysis in liver cirrhosis identified increased hypogalactosylation and increased modification of the serum N-glycome with a bisecting N-acetylglucosamine.(Callewaert et al 2004) Log ratios of different glycans could with good sensitivity and specificity discriminate between early fibrosis and cirrhosis. A follow-up study showed that undergalactosylation was due to the IgG-derived glycan fraction.(Vanderschaeghe et al 2009) This is in line with our data that did not show undergalactosylation in any sample. Also, no glycans were observed within our cohort with a bisecting N-acetylglucosamine. This might be explained by protein specific glycosylation.

4.3 Strengths and limitations

One strength of our study was that we included a wide range of liver disease patients for analysis. We took care to include patients with mild liver disease but also those with advanced chronic liver

disease in need for LTx. Our efforts led to the establishment of a large sample size of over 1000 liver disease patients which adds to the robustness of the study. Additionally, we were able to screen these samples with high resolution mass spectrometry that provided in depth analysis of the intact transferrin glycoprotein, including fucosylation, loss of galactose and the absence of complete glycans.

A limitation of our study is because of its retrospective character. We had access to collected serum samples but were not in possession of fresh plasma or fibroblasts of patients to perform whole-exome sequencing, run a CDG-panel, or perform functional studies. We detected two patients with an increased trisialoTF isoform. Although the cause for this elevation is unknown we believe that the QTOF-MS analysis ruled out abnormal glycosylation due to a defect in V-ATPase-CDG. However, we cannot completely exclude another, possibly novel, CDG.

Another limitation of our study is the high percentage of exclusion of samples resulting from low abundance of serum TF in the samples. A possible explanation could be that low serum TF is associated with cirrhosis.(Bruns et al 2017; Viveiros et al 2018). Additionally, the effect of long term storage on stability of TF is unknown.

4.4 Recommendations

Differentiating primary from secondary glycosylation defects in patients with liver dysfunction can be challenging. Previously analysis of total plasma N-glycans suggested that hyperfucosylation was increased in a single liver disease patient but not in primary CDG.(Guillard et al 2011) This study expands these findings to a large patient group and thus transferrin hyperfucosylation pattern could guide clinicians in decision making.

Our current research exposes caveats in tIEF as a primary diagnostic step. We show that 26% of tIEF samples were abnormal, but these samples did not show a clear type 2 CDG pattern upon QTOF-MS analysis. An important message is that tIEF screening can be false positive because of liver dysfunction.

We suggest caution when interpreting tIEF result in patients with a suspected CDG and liver disease and recommend to maintain a low-threshold to use advanced glycoanalytic methods, preferably in an expertise center. A clear hyperfucosylated pattern is more suggestive for a secondary cause and loss of galactose more suggestive for a primary glycosylation defect. When only sialylation is decreased we suggest a repeat sample to rule out the involvement of exogenous sialidase and a critical review of the phenotype to rule out a CDG with known hyposialylation.

5. Conclusion

Our screening study did not identify V-ATPase assembly factor defects in a cohort of severe liver disease, but we show that end-stage liver disease is associated with hyperfucosylation of transferrin. We confirm that regular CDG screening with tIEF can be complicated by liver disease, itself is associated with mildly abnormal tIEF profiles

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Conflict of Interest

All authors state no conflict of interest.

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Author contributions

JJ, MvS, JD and DL design the study. JJ, FZ and KH performed the experiments. BvH, HM, AvdB and JD provided samples and collected data. JJ, JD and DL wrote the manuscript. All authors critically reviewed the manuscript.

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Tables

Table 1. Patient characteristics

	tIEF selected samples (n=961)		QTOF-MS selected samples (n=149)		
	LTx (n=511)	CLD (n=450)	LTx (n=76)	CLD (n=34)	HC (n=39)
Mean age (years)	48.5 (SD 12.2)	50.0 (SD 15.5)	52.6 (SD 12.0)	51.8 (SD 13.2)	47.1 (SD 14.2)
Male sex	283 (55.4%)	211 (46.9%)	50 (65.8%)	15 (44.1%)	21 (53.8%)
Etiology					
Acute liver failure	38 (7.4%)	2 (0.4%)	1 (1.3%)	0	n.a.
Alcohol liver disease	121 (23.7%)	20 (4.4%)	35 (46.1%)	6 (17.6%)	n.a.
Auto-immune hepatitis	39 (7.6%)	152 (33.8%)	3 (3.9%)	15 (44.1%)	n.a.
Cholestatic liver disease	157 (30.7%)	42 (9.3%)	11 (14.5%)	0	n.a.
Cryptogenic cirrhosis	54 (10.6%)	20 (4.4%)	10 (13.2%)	1 (2.9%)	n.a.
Metabolic disease	26 (5.1%)	7 (1.6%)	7 (9.2%)	0	n.a.
NASH	10 (2.0%)	36 (8.0%)	0	3 (8.8%)	n.a.
Other	61 (11.9%)	36 (8.0%)	8 (10.5%)	2 (5.9%)	n.a.
Unknown	5 (1.0%)	5 (1.1%)	1 (1.3%)	0	n.a.
Gilbert	-	25 (5.6%)	0	2 (5.9%)	n.a.
Viral hepatitis	-	94 (20.9%)	0	5 (14.7%)	n.a.
DILI	-	11 (2.4%)	0	0	n.a.

LTx = Liver transplant recipient, CLD = Chronic liver disease, NASH = non-alcoholic steatohepatitis, DILI = drug induced liver injury. HC = healthy controls, SD= standard deviation, n.a. = not applicable

Table 2. medians of the different tiEF TF isoforms

TF Isoform	Abnormal samples (n=247)					
	Range (%)	Median (%)	SD	Median (%)	SD	MWU p-value
Asialo	0.0-3.2	0.94	1.02	1.24	0.79	0.001
Mono	0.0-5.0	4.84	2.52	2.02	1.74	0.000
Di	3.3-7.6	5.25	1.21	5.27	1.39	0.588
Tri	4.9-10.6	10.40	3.07	11.84	2.25	<0.001
Tetra	47.3 – 62.7	52.63	6.71	52.67	5.72	0.476
Penta	18.7 - 31.5	20.78	3.94	19.95	3.44	0.038

TF=Transferrin, CLD=chronic liver disease, LTx=liver transplantation, MWU: Mann Whitney U test, SD=standard deviation

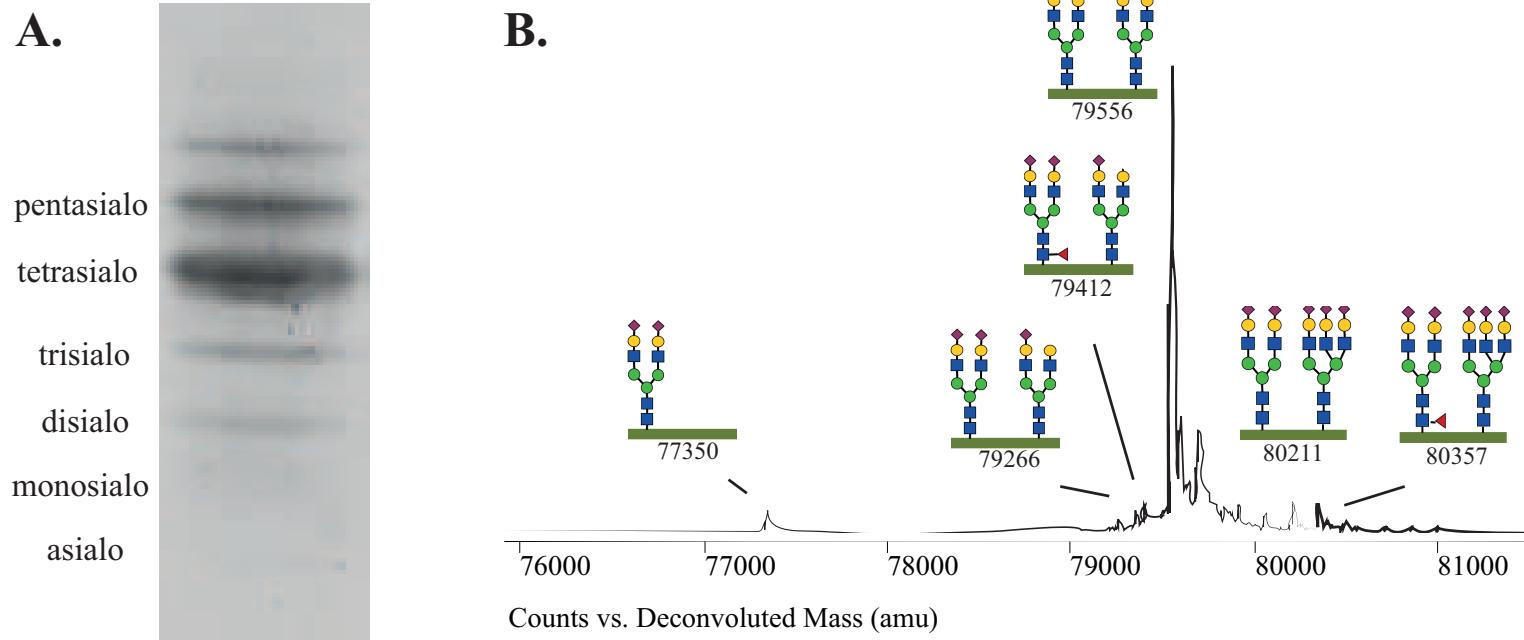
Figure legends

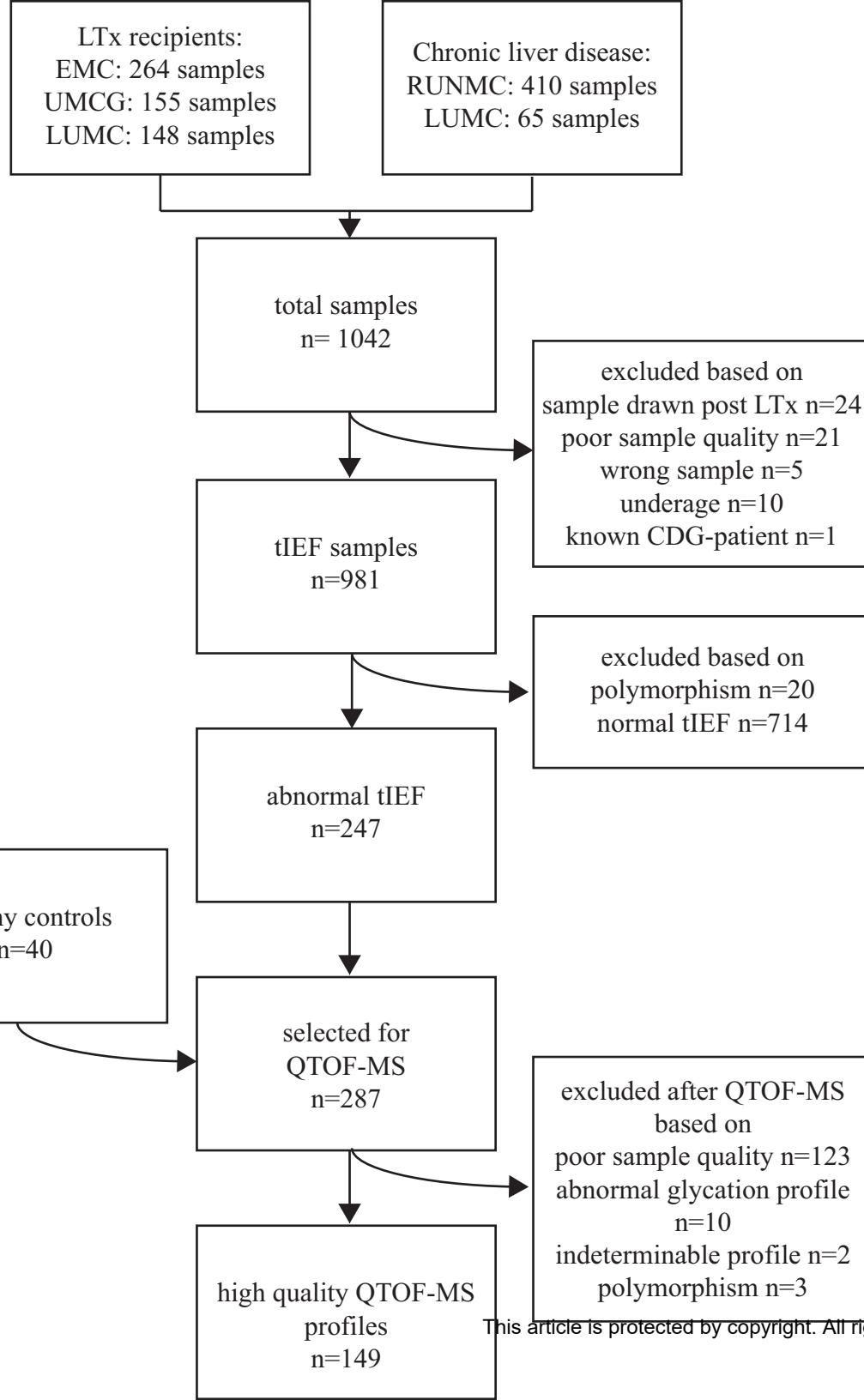
Figure 1. Overview of a normal tIEF and QTOF-MS profile. A. Typical tIEF pattern. The most abundant fraction correlates with the intact TF glycoprotein. B Typical QTOF-MS profile of intact TF with two attached glycans. Shown are the most commonly encountered glycans. The green horizontal bar corresponds with the amino acid backbone. The peak at 79556 amu correlates with the intact TF glycoprotein. The legend for the monosaccharides is: blue square: N-acetylglucosamine, red triangle: fucose, green circle: mannose, yellow circle: galactose, purple diamond: sialic acid.

Figure 2. Flowchart of the study design.

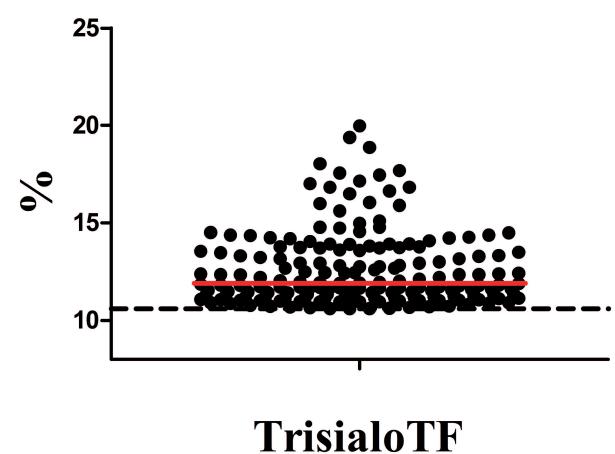
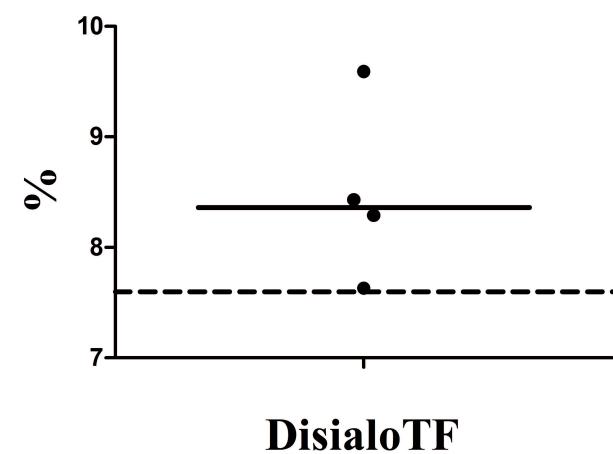
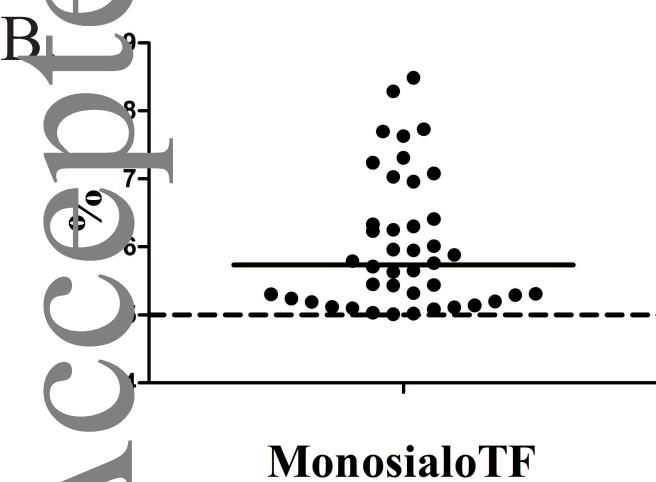
Figure 3. Abnormal tIEF result. A. Waffle chart that shows the distribution of the abnormal tIEF samples. B. Individual medians for the abnormal samples per TF isoform. The dotted line represents upper limit of normal based on internal standards.

Figure 4. Boxplots of the fucosylation ratio of the tri- and pentasialo QTOF-MS isoforms. The left graph shows the fucosylation ratio of trisialotransferrin, or peak 79266. The right graph shows the fucosylation ratio of pentasialotransferrin, or peak 80211. We used a Kruskal-Wallis test for calculation of p-values. HC=healthy controls, CLD=chronic liver disease, LTx=liver transplantation





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disialoTF (2%)
multiple (11%)
monosialoTF (17%)
trisialoTF (70%)

