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Glycome Patterns of Perfusate in Livers Before Transplantation Associate With Primary Non-function

Short title : Perfusate glycomics analysis and PNF

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Abbreviations :

ALT : alanine aminotransferase

AST: aspartate aminotransferase

ATP: adenosine triphosphate

CIT : Cold Ischemia Time

DCD : Donation after Cardiac Death

DRI : Donor Risk Index

EAD : early allograft dysfunction

ECD: extended criteria donor

ET : Eurotransplant

HA: Hyaluronic Acid

HCC: hepatocellular carcinoma

HBV: hepatitis B virus

HCV: hepatitis C virus

HTK : Histidine-Tryptophane-Ketoglutarate

INR: International Normalized Ratio

LDH: lactate dehydrogenase

LT: liver transplantation

MP: Machine perfusion

NASH: non alcoholic steatohepatitis

OR: odds ratio

PNF : primary non function

PSC: primary sclerosing cholangitis

UW : University of Wisconsin Solution

WIT : Warm Ischemia Time

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Key words : Glycan, glycomics, primary non function, perfusate

I. ABSTRACT

Background & Aims: Primary non-function (PNF) is a rare complication after liver transplantation that requires urgent re-transplantation. PNF is associated with livers from extended-criteria donors. Clinical and biochemical factors have not been identified that reliably associate with graft function after liver transplantation. Serum patterns of N-glycans associate with changes in the liver. We analyzed perfusate from grafted liver to identify protein glycosylation patterns associated with PNF.

Methods: We performed a prospective study of consecutive patients who underwent liver transplantation (66 patients, from 1 center, in the derivation set and 56 patients, from 2 centers, in the validation set) in Belgium, from October 1, 2011 through July 31, 2013. All donor grafts were transported using cold static storage, and perfusate samples were collected from the livers by flushing of hepatic veins before transplantation. Protein-linked N-glycans were isolated from perfusate samples and analyzed with a multi-capillary electrophoresis-based ABI3130 sequencer.

We compared glycan patterns between patients with vs without PNF of transplanted livers. PNF was defined as the need for urgent re-transplantation when a graft had no evidence of function, after exclusion of other causes such as hepatic artery thrombosis or acute cellular rejection.

Results: The relative abundance of a single glycan, agalacto core-alpha-1,6-fucosylated biantennary glycan (NGA2F) was significantly increased in perfusate of livers given to 4 patients who developed PNF after liver transplantation compared to livers given to patients who did not develop PNF. Level of NGA2F could identify patients with PNF with 100% accuracy. This glycomarker was the only factor associated with PNF in multivariate analysis in the derivation and the validation sets ($P < .0001$).

Conclusions: In an analysis of patients who underwent liver transplantation, we associated graft perfusate level of the glycosylated protein NGA2F with development of PNF with 100% accuracy, and validated this finding in a separate cohort of patients. This biomarker might be used to assess grafts before transplantation—especially when high-risk organs are under consideration.

KEY WORDS: liver failure, glycomics, secretion, hepatocyte

Graphical Abstract : NGA2F is increased in perfusate of patients who developed primary non function.

II. INTRODUCTION

Liver transplantation is the treatment of choice for end-stage liver disease, acute liver failure and selected patients with hepatocellular carcinoma¹, with excellent long term results². A major challenge in the expansion of organ transplantation is shortage of donor organs, which is reflected in a unacceptably high waiting list mortality³. This bottleneck has led to the use of expanded criteria donors (ECD) donors in order to increase the donor pool⁴⁻⁶. These ECD livers carry an increased risk of liver failure or an increased risk of disease transmission. Scoring systems have been developed to predict the organ quality based on clinical characteristics of the donor including the donor risk index (DRI)⁷, and the more recently developed European variant, the Eurotransplant donor risk index (ET-DRI)⁸. However, although these instruments are of great value for population based studies, they fail to predict the outcome of a single donor⁹ and cannot yet be included in allocation strategies.

A particular problem associated with the use of these low quality grafts is primary non function (PNF)¹⁰. PNF occurs after 2-10%¹¹⁻¹³ of liver transplantations and was first described by Shaw et al.¹⁴ as a situation where “a graft never demonstrated evidence of initial function following transplantation” after exclusion of technical causes and acute cellular rejection needing. These PNF patients need an urgent retransplantation. A more frequent but less dramatic phenomenon is early allograft dysfunction (EAD), which is a functional insufficiency after orthotopic liver transplantation characterized by the appearance of hyperbilirubinemia, coagulopathy, encephalopathy or ascites¹⁵. It occurs in up to 1 in 4 liver transplantations¹⁵. In contrast to PNF, it does not require urgent retransplantation. Nevertheless, EAD is associated with increased graft loss and patient mortality within the first 90 days^{15,16} after liver transplantation.

Reliable prognostic clinical scores or biomarkers that could predict the appearance of PNF or EAD after LT before the LT is performed, are not yet available. Here, we performed a comprehensive

analysis of protein-linked, N-glycans in perfusate in relation to the appearance of PNF and EAD after LT.

During the last ten years it has become clear that N-glycan analysis of serum, also called clinical glycomics, is useful for the diagnosis of various liver diseases¹⁷. These biomarkers are based on the concept that the majority of human proteins are glycosylated, which is a highly regulated and specific enzymatic process occurring in the endoplasmic reticulum and the Golgi body¹⁸. Interestingly, the majority of serum proteins are secreted by hepatocytes, supporting the concept that the serum glycomic content reflects (patho)physiologic changes in the liver. Another smaller source of serum glycans are B-cells producing IgG molecules, which are also glycosylated, in particular on a conserved site in the CH₂ domain of IgG^{19,20}. This concept led to the development of glycomic based serum biomarkers for the diagnosis of liver fibrosis^{21,22}, cirrhosis^{23,24} and hepatocellular carcinoma (HCC)^{25,26} and a biomarker that can distinguish simple liver steatosis from non-alcoholic steatohepatitis^{27,28}.

The aim of this study was to explore the feasibility of N-glycan analysis of glycoproteins in graft perfusate after flushing of the donor liver graft immediately before transplantation. The second aim was to search for distinct N-glycan profiles in patients who developed PNF or EAD compared to patients with good early liver graft function, in order to develop biomarkers to monitor organ quality and predict PNF.

The results of the present study, with a test cohort from one clinical center and an independent validation cohort from two centers, provide a strong proof of concept that the analysis of N-glycans in preservation fluid can predict the appearance of PNF after liver transplantation.

III. PATIENTS AND METHODS

PATIENTS

Consecutive patients undergoing liver transplantation at the liver transplant unit of Ghent University Hospital (Belgium) were included in the study. The test cohort prospectively included patients between October 1st 2011 and July 31th 2013. The results from the test cohort were validated in prospective multicenter Belgian cohort including consecutive patients from the liver transplant unit of Ghent University Hospital (between 1 September 2013 and 30 April 2017) and the liver transplant unit of the University Hospitals Leuven (between 1 August 2016 and 28 February 2017).

PNF was defined as the need for urgent retransplantation when a graft never demonstrated any evidence of initial function following transplantation after exclusion of other causes like hepatic artery thrombosis or acute cellular rejection. These patients developed high transaminases, low PT, high bilirubin and high lactate within 24 hours after liver transplantation.

EAD was defined according to Olthoff et al.¹⁵ as the presence of one or more of the following previously defined postoperative laboratory analyses : bilirubin ≥ 10 mg/dl on day 7, INR ≥ 1.6 on day 7, alanine or aspartate aminotransferases (AST or ALT) > 2000 UI/l within the first 7 days.

Donor risk index was calculated according to Feng et al.⁷.

SAMPLE COLLECTION AND PROCESSING

The perfusate sample was obtained during the back-table preparation of the graft at arrival in the transplant hospital. Liver grafts were procured according to local practices and transported to our center in Histidine-tryptophan-ketoglutarate (Custodiol HTK, Franz Köhler Chemie GmbH, Bensheim, Germany), Wisconsin solution (UW Belzer, Costorsol, Bridge to Life, London, United Kingdom) or Institut Georges Lopez solution (IGL-1, Institut Georges Lopez, SAS, Lissieu, France). During the back-table procedure, the left hepatic vein was flushed with a syringe of 20 ml of perfusate procured in the container in which the graft had been transported under normal hydrostatic pressure. The

perfusate exiting the hepatic veins after this vein flush were collected and immediately stored at -21°C.

N-GLYCAN ANALYSIS

All samples were defrosted and analysed in two runs, one for the test cohort and one for the validation cohort. The glycomic analysis was performed in duplo. Perfusate samples were prepared using the 96 well on-membrane deglycosylation method, which has been described in detail previously²⁹. In summary, N-glycans bound to proteins in 500 µl of preservation solution were denatured using 1 ml of denaturing buffer (10 mM NH₄HCO₃ pH 8.3, containing 3.5% SDS). Then, N-glycans were released from the proteins, using Peptide N-glycosidase F (PNGase F- provided by the laboratory of Prof. Nico Callewaert, VIB-Ugent Center for Medical Biotechnology, Belgium) and labeled with 8-aminopyrene-1,3,6-trisulphonic acid (APTS - Molecular Probes, Eugene, OR,USA). Subsequently, the glycans were desialylated overnight at 37° C by the addition of 2 µl of 10mM ammonium acetate pH 5.0 containing 40 mU of *Arthrobacter ureafaciens*-2,3/6/8-sialidase (also provided by the laboratory of Prof. Nico Callewaert). Samples were resuspended to 10 µl with ultrapure water. Two microliters of the desialylated N-glycan samples were analyzed with a multicapillary electrophoresis-based ABI3130 sequencer.

A desialylated serum protein N-glycan profile was obtained for the study objects. We quantified 13 peaks that were detectable in all samples using Peak Scanner 2 Version 2.0 software (Applied Biosystems, Foster City, CA, USA), and normalized their abundance to the total peak height intensity (Figure 1). The nature of these peaks has been identified before by structural analysis²³.

AST and ALT levels were measured in the perfusate using a Cobas c701 analyzer (Roche) after addition of pyridoxalphosphate.

STATISTICAL ANALYSIS

Statistical analysis was performed using IBM®SPSS®Statistics Version 22.0. Continuous variables were compared between the group of patients who developed PNF, EAD and those who did not using a Kruskal-Wallis test. Comparison of two non-parametric variables was performed using Mann Whitney U test. The prognostic value of potential biomarkers was assessed using exact logistic regression. Multivariate analysis was performed using a multivariate logistic regression model. Statistical significance was set at the alpha level = 0.05.

ETHICS

The study conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the ethical committee of the participating centers.

IV. RESULTS

PATIENTS CHARACTERISTICS

In the test cohort, 66 liver transplantation were performed in 64 patients (Table 1). Three patients developed PNF, 19 EAD and 44 patients showed a normal postoperative evolution of liver function. In the non-PNF group, 2 more patients needed an urgent early retransplantation, both due to a hepatic artery thrombosis, and were thus not considered as PNF patients. Recipient and donor characteristics are summarized in Table 1. The major indication for liver transplantation was cirrhosis due to alcohol (n=22), chronic hepatitis C virus (HCV) infection (6), chronic hepatitis B virus (HBV) infection (n=3), autoimmune hepatitis (n=2) or non alcoholic steatohepatitis (NASH) (n=4). Other indications were primary sclerosing cholangitis (PSC) (n=4), polycystic liver disease (n=5), acute liver failure (n=9), familial hyperoxaluria (n=1), epitheloid hemangioendothelioma (n=1), ischemia type biliary lesions in the transplant liver (n=2), veno-occlusive disease (n=1) and adenomatosis (n=2).

The validation cohort includes 56 patients from Ghent University Hospital (n=39) and University Hospitals Leuven (n=20) (Table 1). Recipient and donor characteristics are summarized in Table 1. One patient developed PNF, 19 EAD and 36 patients showed a normal postoperative evolution of liver function. In the validation cohort, indications for liver transplantation were decompensated cirrhosis (n=27), hepatocellular carcinoma (HCC) (n=18), ALF (n=1), PSC (n=6), polycystic liver disease (n=3), cystic fibrosis (n=1).

GLYCOMIC ANALYSIS OF PERFUSATE

The technique used for N-glycan analysis is based on a DNA sequencer-assisted fluorophore-assisted capillary electrophoresis that was initially developed and validated for serum analysis²¹ and has been used for urine analysis²². Now, this technique was applied on perfusate from liver transplant patients. Using the standard protocol, we obtained an electropherograms with 13 peaks (figure 1), very similar with regard to N-glycan composition as what has been observed in serum and urine^{23,29}.

PROGNOSTIC PERFORMANCE OF PERFUSATE GLYCOMICS FOR EAD AND PNF

We quantified 13 peaks that were well detectable in all samples and normalized their abundance to the total peak height intensity per patient (Table 2). Peak 1, 2, and 3 showed discriminative changes according to development of PNF (Figure 1 and 2). However, as can be appreciated from Figure 2, for EAD patients a specific glycomic signature was not apparent. For further analysis, we focused on PNF and pooled the EAD and non-PNF patients.

A more detailed analysis disclosed that the perfusate N-glycan profile of PNF patients was highly discriminative from non-PNF patients based on the increased abundance of peak 1, an agalacto, core-alpha-1,6-fucosylated biantennary structure (NGA2F) (Figure 2) compared to the EAD and non-PNF/EAD patients ($p < 0.0001$) with an accuracy of 100% using a cut-off of 13%.

Using exact logistic regression (univariable analysis), the odds ratio (OR) for PNF development for an increase of peak 1 with an absolute value of 1 was 1.801 ($p < 0.0001$; 95% CI 1.321-infinity).

The very pronounced increase of this first glycan was confirmed in the single PNF patient in the validation cohort. Based on the same cut-off of 13%, the PNF patient could be identified among the non-PNF patients.

In one patients with PNF we were able to analyse the serum sample of the liver donor. Interestingly, the donor sample did not show an increased abundance of our glycomarker, in contrast to the perfusate of the same patient that showed a significant increase.

PROGNOSTIC PERFORMANCE OF PERFUSATE AST AND ALT FOR EAD AND PNF

AST and ALT levels were determined in perfusate. Comparable levels of AST ($p = 0.661$) and ALT levels ($p = 0.617$) were found in PNF, EAD and other patients.

A PERFUSATE GLYCOMIC BIOMARKER AS PREDICTOR OF PNF IN LIVER TRANSPLANTATION

After establishing a perfusate glycomic signature predictive of PNF development following liver transplantation, we analyzed whether other donor- or recipient parameters were predictive for the occurrence of PNF using an univariate analysis (Mann Whitney U test). In both cohorts, none of the clinical donor parameters were predictive for the development of PNF after liver transplantation (Table 3). Analysis of AST and ALT levels in perfusate was only available in the test cohort but was not increased in the case of PNF development (Table 3). In a multivariate logistic regression model (Table 4) including our glycomic marker (NGA2F), the donor risk index (DRI) and AST and ALT in perfusate, only the glycomarker NGA2F was an independent predictor of PNF ($p < 0.0001$).

V. DISCUSSION

We propose a new biomarker based on glycomic analysis of perfusate that is able to identify donor livers at risk for the development of PNF. This biomarker could be a tool to safely select high-risk organs (eg. ECD organs) for liver transplantation that otherwise would be discarded from the donor pool based on a conventional clinical assessment as it is often performed now. Secondly, this glycomic marker could prevent unsuccessful engraftments that will increase the morbidity and mortality following an urgent retransplantation. Indeed, in this cohort, not any clinical parameter was able to predict PNF, in contrast to the glycomarker that could accurately differentiate between PNF and non-PNF patients in these cohorts. To our knowledge, not a single biomarker has demonstrated the same accuracy today.

Perfusate biomarkers are an attractive alternative for liver biopsy or serum markers, because perfusate is believed to represent the condition of the entire liver parenchyma and is easy to collect in large volumes. However, only few perfusate markers have previously shown any utility in the prediction of PNF³⁰. Conventional transaminases and lactate dehydrogenase (LDH) are increased in the perfusate of patients developing PNF and EAD, reflecting hepatocellular injury. Unfortunately, high enzyme activity in the effluent did not necessarily predict a malfunction of the graft, as the specificity for PNF was only 42%, making it a sensitive marker for safe livers at a cost of excluding many potential good grafts³¹. In our cohort, AST and ALT levels showed a wide range among all patients, without distinct correlation with EAD or PNF. Xanthine, a purine nucleotide, was shown to be increased in the effluent perfusate of PNF patients, but was not able to discriminate between the patients of both groups in a clinical study³². In the same patients, adenine nucleotides (adenoside triphosphate (ATP), adenoside diphosphate (ADP), and adenoside monophosphate (AMP)) were decreased in liver tissue after transplantation, reflecting irreversible injury and necrosis of the liver and the retardation of the recovery of cellular (ATP) after reperfusion^{33,34}. The group of Starzl showed 20 years ago that hyaluronic acid (HA) levels higher than 400 µg/L in perfusate are a strong predictor of PNF. HA is a glycosaminoglycan catabolized by the liver hepatocyte microvasculature. In a cohort

of 70 patients, 6 PNF patients were correctly classified as PNF, but another 6 patients, presumed to develop PNF based on the 400 $\mu\text{g/L}$ cut-off, showed a normal clinical outcome³⁵. Similar findings were obtained in a validation study³⁶, again positioning the test as a marker for identifying safe livers.

The proposed glycomarker for PNF prediction managed to identify the 4 patients who developed PNF after liver transplantation by an analysis that could be performed before the liver transplantation, without false positives. It is based on the increased abundance of one single undergalactosylated biantennary glycan (NGA2F). Undergalactosylated biantennary glycans were previously shown to be exclusively present on IgG²¹, relating it to inflammatory states. Although the pathophysiology of PNF is complex, extensive ischemic- and preservation injury has been recognized as a main pathophysiological driver^{37,38}. IgG undergalactosylation is a marker of necroinflammation also found in patients with rheumatoid arthritis³⁹ and increasing levels of liver fibrosis²¹. In this cohort, in PNF and non PNF patients comparable levels of IgG concentration in perfusate were measured, highlighting that the increased abundance of NGA2F in PNF patients is not related to a potential increase of total perfusate IgG but to specific glycomic alterations. The single measurement of peak 1 is a powerful representation of the rise of the other two undergalactosylated glycans (NGA2FB and NG1A2F) in the perfusate protein N-glycome and is believed to represent inflammatory disturbances in the donor liver that will fail after transplantation.

The accuracy for the prediction of PNF was extremely high (100%) in the cohorts studied here. On the other hand we found no clear relationship between the perfusate glycomic profile and the occurrence of EAD. We speculate that this may be related to the fact that EAD is a complex and multifactorial syndrome that is not only related to the quality of the donor liver graft but also to recipient characteristics and intra- and postoperative events. These parameters will of course not be captured in the pretransplant glycomic profile. Interestingly, 2 patients who needed an urgent retransplantation of the liver due to hepatic artery thrombosis showed an unremarkable glycomic

profile (normal profile in Figure 1). On the other hand, PNF is mainly related to very low quality grafts which can be captured quite convincingly by the glycomic profile.

Concerns could be raised that the glycomarker measurement in perfusate is a mere contamination of donor serum. The different N-glycomic profile of the donor serum and the perfusate in a PNF patient provide a strong argument against this hypothesis.

Machine perfusion (MP) is a new approach in liver transplantation to improve graft preservation of ECD grafts. There is a huge need for biomarkers that can evaluate the quality of the liver graft during machine perfusion³⁰, and the role of this glycomarker needs to be explored in this setting as well.

A drawback in the application of this technique is the turnover time that requires 48 hours. However, sample preparation for serum samples has recently been highly simplified and sped up to a turnaround time of 2 hours (Callewaert et al., unpublished results) in preparation for clinical implementations, and the analysis can be performed on widely available high-throughput capillary electrophoresis platforms including those that are in use in clinical genetics (capillary DNA sequencers) and clinical chemistry laboratories (Helena V8 nexus). Implementation of the technique for perfusate on these clinical-analytics methods is ongoing, which will reduce the turnover time to a clinically acceptable time frame.

In conclusion, the study provides a strong proof-of-concept and validation that the glycomic analysis of perfusate predicts with very high accuracy (100% in our cohorts) the occurrence of PNF and could answer a real medical need in the field of liver transplantation. These findings need to be further validated in larger multi-center studies in the upcoming years.

References

1. European Association for the Study of the Liver. Electronic address: easloffice@easloffice.eu.

- EASL Clinical Practice Guidelines: Liver transplantation. *J Hepatol* 2016;64:433–85.
2. Adam R, Karam V, Delvart V, et al. Evolution of indications and results of liver transplantation in Europe. A report from the European Liver Transplant Registry (ELTR). *J Hepatol* 2012;57:675–688.
 3. Branger P, Samuel U. *Eurotransplant Annual Report 2015*. 2015.
 4. Durand F, Renz JF, Alkofer B, et al. Report of the Paris consensus meeting on expanded criteria donors in liver transplantation. *Liver Transpl* 2008;14:1694–707.
 5. Alkofer B, Samstein B, Guarrera J V, et al. Extended-donor criteria liver allografts. *Semin Liver Dis* 2006;26:221–33.
 6. Foster R, Zimmerman M, Trotter JF. Expanding donor options: marginal, living, and split donors. *Clin Liver Dis* 2007;11:417–29.
 7. Feng S, Goodrich NP, Bragg-Gresham JL, et al. Characteristics associated with liver graft failure: the concept of a donor risk index. *Am J Transplant* 2006;6:783–90.
 8. Braat AE, Blok JJ, Putter H, et al. The Eurotransplant donor risk index in liver transplantation: ET-DRI. *Am J Transplant* 2012;12:2789–96.
 9. Reichert B, Kaltenborn A, Goldis A, et al. Prognostic limitations of the Eurotransplant-Donor Risk Index in liver transplantation. *J Negat Results Biomed* 2013;12:18.
 10. Ploeg RJ, D'Alessandro a M, Knechtle SJ, et al. Risk factors for primary dysfunction after liver transplantation - a multivariate analysis. *Transplantation* 1993;55:807–813.
 11. Busuttil RW, Shaked A, Millis JM, et al. One thousand liver transplants. The lessons learned. *Ann Surg* 1994;219:490-7-9.
 12. D'Alessandro AM, Ploeg RJ, Knechtle SJ, et al. Retransplantation of the liver--a seven-year

- experience. *Transplantation* 1993;55:1083–7.
13. Mimeault R, Grant D, Ghent C, et al. Analysis of donor and recipient variables and early graft function after orthotopic liver transplantation. *Transplant Proc* 1989;21:3355.
 14. Shaw BW, Gordon RD, Iwatsuki S, et al. Hepatic Retransplantation. *Transplant Proc* 1985;17:264–271.
 15. Olthoff KM, Kulik L, Samstein B, et al. Validation of a current definition of early allograft dysfunction in liver transplant recipients and analysis of risk factors. *Liver Transpl* 2010;16:943–9.
 16. Pomposelli JJ, Goodrich NP, Emond JC, et al. Patterns of Early Allograft Dysfunction in Adult Live Donor Liver Transplantation: The A2ALL Experience. *Transplantation* 2016;100:1490–9.
 17. Blomme B, Steenkiste C Van, Callewaert N, et al. Alteration of protein glycosylation in liver diseases. *J Hepatol* 2009;50:592–603.
 18. Taylor M, Drickamer K. N-linked glycosylation. In: *Introduction to Glycobiology.*; 2011:17–31.
 19. Callewaert N, Contreras R, Mitnik-Gankin L, et al. Total serum protein N-glycome profiling on a capillary electrophoresis-microfluidics platform. *Electrophoresis* 2004;25:3128–3131.
 20. Vanderschaeghe D, Szekrényes A, Wenz C, et al. High-throughput profiling of the serum N-glycome on capillary electrophoresis microfluidics systems: toward clinical implementation of GlycoHepatoTest. *Anal Chem* 2010;82:7408–15.
 21. Vanderschaeghe D, Laroy W, Sablon E, et al. GlycoFibroTest is a highly performant liver fibrosis biomarker derived from DNA sequencer-based serum protein glycomics. *Mol Cell Proteomics* 2009;8:986–94.
 22. Kuno A, Ikehara Y, Tanaka Y, et al. A serum “sweet-doughnut” protein facilitates fibrosis

- evaluation and therapy assessment in patients with viral hepatitis. *Sci Rep* 2013;3:1065.
23. Callewaert N, Vlierberghe H Van, Hecke A Van, et al. Noninvasive diagnosis of liver cirrhosis using DNA sequencer-based total serum protein glycomics. *Nat Med* 2004;10:429–34.
 24. Verhelst X, Vanderschaeghe D, Castéra L, et al. A glycomics-based test predicts the development of hepatocellular carcinoma in cirrhosis. *Clin Cancer Res* 2017;23:2750–2758. 2016].
 25. Liu X-E, Desmyter L, Gao C-F, et al. N-glycomic changes in hepatocellular carcinoma patients with liver cirrhosis induced by hepatitis B virus. *Hepatology* 2007;46:1426–1435.
 26. Debruyne EN, Vanderschaeghe D, Vlierberghe H Van, et al. Diagnostic value of the hemopexin N-glycan profile in hepatocellular carcinoma patients. *Clin Chem* 2010;56:823–31.
 27. Blomme B, Fitzpatrick E, Quaglia a., et al. Serum protein N-glycosylation in paediatric non-alcoholic fatty liver disease. *Pediatr Obes* 2012;7:165–173.
 28. Blomme B, Francque S, Trépo E, et al. N-glycan based biomarker distinguishing non-alcoholic steatohepatitis from steatosis independently of fibrosis. *Dig Liver Dis* 2012;44:315–22.
 29. Laroy W, Contreras R, Callewaert N. Glycome mapping on DNA sequencing equipment. *Nat Protoc* 2006;1:397–405.
 30. Verhoeven CJ, Farid WRR, Jonge J de, et al. Biomarkers to assess graft quality during conventional and machine preservation in liver transplantation. *J Hepatol* 2014;61:672–684.
 31. Lange R, Erhard J, Rauen U, et al. Determination of hepatocellular enzymes in effluent of human liver grafts for preoperative evaluation of transplant quality. *Transplantation* 1996;62:1255–9.
 32. Hamamoto I, Takaya S, Todo S, et al. Can adenine nucleotides predict primary nonfunction of

- the human liver homograft? *Transpl Int* 1994;7:89–95.
33. Marubayashi S, Takenaka M, Dohi K, et al. Adenine nucleotide metabolism during hepatic ischemia and subsequent blood reflow periods and its relation to organ viability. *Transplantation* 1980;30:294–6.
34. Harvey PR, Lu S, McKeown CM, et al. Adenine nucleotide tissue concentrations and liver allograft viability after cold preservation and warm ischemia. *Transplantation* 1988;45:1016–20.
35. Bronsther OL, Rao PN, Pinna A, et al. Effluent levels of hyaluronic acid can predict ultimate graft outcome after clinical liver transplantation: a prospective series. *Transplant Proc* 1993;25:1538–40.
36. Rao PN, Bronsther OL, Pinna AD, et al. Hyaluronate levels in donor organ washout effluents: a simple and predictive parameter of graft viability. *Liver* 1996;16:48–54.
37. Varotti G, Grazi GL, Vetrone G, et al. Causes of early acute graft failure after liver transplantation: analysis of a 17-year single-centre experience. *Clin Transplant* 2005;19:492–500.
38. Op den Dries S, Sutton ME, Lisman T, et al. Protection of bile ducts in liver transplantation: looking beyond ischemia. *Transplantation* 2011;92:373–9.
39. Beneden K Van, Coppieters K, Laroy W, et al. Reversible changes in serum immunoglobulin galactosylation during the immune response and treatment of inflammatory autoimmune arthritis. *Ann Rheum Dis* 2009;68:1360–5.

Figure 1. The structures of the N-glycan peaks in a human perfusate sample. A. The structures of the N-glycan peaks in a human perfusate sample as obtained using capillary electrophoresis yields 13 peaks. From left to right : Peak 1 is an agalacto, core-alpha-1,6-fucosylated biantennary (NGA2F), peak 2 is an agalacto, core-alpha-1,6-fucosylated bisecting biantennary (NGA2FB), peak 3 and peak 4 are single agalacto, core-alpha-1,6-fucosylated biantennary structures (NG1A2F), peak 5 is the bigalacto biantennary glycan NA2, peak 6 is the bigalacto, core-alpha-1,6-fucosylated biantennary glycan NA2F, peak 7 is the bigalacto, core-alpha-1,6-fucosylated bisecting biantennary glycan NA2FB, peak 8 is the triantennary glycan NA3, peak 9 is the branching alpha-1,3-fucosylated triantennary glycan NA3Fb, peak 10 is the core-alpha-1,6-fucosylated triantennary glycan NA3Fc, peak 11 is the branching alpha-1,3-fucosylated and core alpha-1,6-fucosylated triantennary glycan NA3Fbc, peak 12 is a tetra-antennary (NA4) and peak 13 is a branching alpha-1,3-fucosylated tetra-antennary (NA4Fb) glycan. The symbols used in the structural formulas are: square indicates beta-linked N-acetylglucosamine (GlcNAc); yellow circle indicates beta-linked galactose, triangle indicates alpha/beta-1,3/6-linked fucose; green circle indicates alpha/beta-linked mannose.

B. This graphs shows the perfusate analysis of a patient without PNF (upper panel) and a patient who developed PNF (lower panel). Note the increased abundance of peak 1, 2 and 3 (green). The increase of peak 1 alone is discriminative of PNF versus no-PNF.

Figure 2. Overview of the relative abundance of relevant glycans in patients that developed PNF

afterward. Peak 1 (NGA2F) shows 100% accuracy for prediction of PNF. P values were calculated

using Mann Whitney U- test. * $p < 0.05$, ** $p < 0.001$

Table 1. Patient Characteristics

Patients were divided according to the occurrence of PNF or EAD. Variables between groups were compared using Kruskal-Wallis test. Continuous variables are expressed as means followed by standard deviation (SD) between brackets. Variables are expressed as means followed by standard deviation between brackets. *Highest INR value between postoperative day 2 and day 7. **Highest total bilirubin value between postoperative day 2 and 7

	Test Cohort (n=66)				Validation Cohort (n=56)			
	No PNF/EAD (n =44)	EAD (n=19)	PNF (n=3)	p-value	No PNF/EAD (n =36)	EAD (n=19)	PNF (n=1)	p-value
Recipient Characteristics								
Age	49.68 (13.64)	52 (15.09)	50 (19.16)	0.704	53.37 (15.84)	55.42 (12.42)	68	0.303
Sex (M/F)	26/18	13/6	2/1				1/0	
AST at 48 hours (U/L)	594 (446)	2194 (2138)	5488 (6195)	0.001	888.94 (488.91)	4279.16 (2674.85)	5954	<0.001
ALT at 48 hours (U/L)	592 (456)	1574 (1461)	1384 (1271)	0.004	592 (296)	2649 (1492)	4054	<0.001
INR*	1.58 (0.76)	2.17 (1.13)	3.52 (0.96)	0.001	1.91 (1.02)	1.31 (0.58)	14	0.218
Total bilirubin** (mg/dl)	2.91 (3.06)	6.06 (6.55)	11.73 (8.40)	0.006	1.85 (1.63)	1.84 (1.45)	10	0.205
MELD	19.16 (10.03)	18.42 (9.80)	30 (15.62)	0.360	15.94 (7.47)	13.13 (6.01)	10.86	0.568
Donor Characteristics								
Age	74.89 (17,69)	47.68 (16.88)	47.67 (18.23)	0.989	52.97 (19.02)	52,32 (13.06)	36	0.469
Length (cm)	172.80 (6.75)	174.32 (7.42)	171 (7.8)	0.310	171.86 (8.21)	175.89 (6.97)	185	0.082
Weight (cm)	77.08 (11.76)	84.84 (23.41)	60.50 (6.34)	0.093	72.86	81.42	75	0.011

					(12.90)	(8.85)		
Perfusate (HTK/UW/IGL-1)	33/10/0	12/6/0	2/1/0		12/2/20	9/2/6	1/0/0	
CIT (minutes)	422.18 (121.68)	389.42 (102.338)	493 (9.90)	0.763	561.55 (986.829)	425.94 (128.957)	930	0.213
Implantation time (minutes)	36.73 (12.23)	42.26 (19.36)	40 (11.31)	0.473	33.30 (9.37)	34.73 (10.99)	80	0.227
AST (U/L)	55.43 (51.91)	63.05 (61.85)	22.50 (3.53)	0.705	63.49 (68.81)	53.47 (36.33)	77	0.704
ALT (U/L)	42.08 (39.65)	53.53 (65.67)	25.50 (19.09)	0.611	41.86 (42.20)	62.63 (81.68)	65	0.252
GGT (mg/dl)	69.53 (101.94)	59.26 (44.20)	20.50 (7.79)	0.704	66.46 (85.87)	97.89 (108.20)	29	0.406
Total Bilirubin (mg/dl)	2.43 (5.77)	2.14 (3.85)	0.72 (0.17)	0.925	0.59 (0.41)	7.61 (29.89)	1.27	0.429
DRI	1.67 (0.36)	1.69 (0.41)	1.71 (0.35)	0.897	2.14 (0.95)	1.82 (0.55)	1.43	0.447
DCD	0	3	0		13	6	0	

Table 2. Results. Variables between groups were compared using Kruskal-Wallis test. Continuous variables are expressed as means followed by standard deviation (SD) between brackets.

	Test Cohort (n=66)				Validation Cohort (n=56)			
Perfusate Markers	No PNF/EAD (n =44)	EAD (n=19)	PNF (n=3)	p-value	No PNF/EAD (n =36)	EAD (n=19)	PNF (n=1)	p-value
Peak 1	4.54 (1.87)	4.54 (2.64)	18.44 (6.08)	0.013	3.89 (2.76)	3.28 (1.99)	25.008	0.204
Peak 2	0.97 (0.77)	1.22 (0.55)	2.27 (1.07)	0.020	1.00 (0.65)	0.81 (0.57)	21.96	0.121
Peak 3	3.55 (1.63)	3.26 (1.65)	9.25 (7.08)	0.048	3.08 (1.67)	2.97 (1.53)	1.29	0.351
Peak 4	4.05 (2.33)	5.05 (3.47)	4.70 (2.66)	0.740	16.12 (14.47)	14.64 (10.75)	9.48	0.972
AST (IU/L)	136 (183)	1357 (4490)	60 (50)	0.661				
ALT (IU/L)	50 (109)	806 (2825)	24 (19)	0.617				
IgG	6,5 (4,40)	28,81 (70,26)	3 (3,2)	0.654				

Table 3. Prognostic markers for the development of PNF compared to non-PNF patients (Univariable analysis - Mann Withney U test). Legend: *Highest INR value between postoperative day 2 and day 7. **Highest total bilirubin value between postoperative day 2 and 7 . N/A: not available

	Test Cohort	Validation Cohort
Donor Characteristics	p-value	p-value
Age	0.954	0.109
Sex (M/W)	0.772	0.509
Length (cm)	0.269	0.182
Weight (cm)	0.175	1
Perfusate (HTK/UW/IGL)	0.858	0.265
CIT (minutes)	0.794	0.077
WIT (minutes)	0.518	0.061
AST (U/L)	0.641	0.545
ALT (U/L)	0.908	0.400
GGT (mg/dl)	0.871	0.741
Total Bilirubin (mg/dl)	0.734	0.291
NHBD	0.908	0.473
DRI	0.772	0.500
MELD	0.130	0.842
Perfusate analysis		
AST	0.653	N/A
ALT	0.589	N/A
Peak 1 = NG1A2F	<0.0001	0.037
Peak 2 = NGA2FB	0.042	0.037
Peak 3 = NG1A2F	0.010	0.185

Table 4. Prognostic markers for the development of PNF (Multivariate logistic regression model).

	Test cohort
	p-value
NG1A2F perfusate	<0.0001
AST perfusate	0.800
ALT perfusate	0.818
Donor Risk Index	0.818