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# Serum Glycomics on Postoperative Day 7 Are Associated With Graft loss Within 3

### Months After Liver Transplantation Regardless of Early Allograft Dysfunction

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**Disclosure** Xavier Verhelst: listed as co-inventor on a patent owned by Ghent University (Belgium) for a glycomics-based biomarker for the prediction of primary non function after LT. Hans Van Vlierberghe: listed as co-inventor on a patent owned by Ghent University (Belgium) for a glycomics-based biomarker for the prediction of primary non function after LT. Nico Callewaert: N. Callewaert is listed as a co-inventor on a patent on GlycoCirrhoTest that

is owned by VIB vzw and has been licensed to Helena Biosciences

Other authors have nothing to disclose.

## **Authorship page**

XV, HV Participated in research design

XV, RC, Participated in the writing of the paper

XV, AG, AV, LA, FB, XR Participated in the performance of the research

XV, LM, NC Contributed new reagents or analytic tools

XV, RC, HV Participated in data analysis

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### Abbreviations:

DRI: Donor risk index

DSA-FACE: DNA sequencer associated fluorophore associated capillary electrophoresis

EAD: early allograft dysfunction

ECD: extended criteria donors

ET: eurotransplant

HCC : hepatocellular carcinoma

IVG: indocyanine green

LT: liver transplantation

NASH: non alcoholic steatohepatitis

NPV: negative predictive value

PPV: positive predictive value

#### ABSTRACT

Background: Prediction of outcome after LT is limited by the lack of robust predictors of graft failure. In this prospective study, we aimed to define a serum glycomic signature in the first week after liver transplantation (LT) that is associated with graft loss at 3 months after LT. Methods: Patients were included between 1 January 2011 and 28 February 2017. Glycomic analysis was performed using DNA sequencer associated fluorophore associated capillary electrophoresis (DSA-FACE) on a serum sample 1 week after LT. Making use of Lasso regression, an optimal glycomic signature was identified, associated with 3 months graft survival.

Results: In this cohort of 131 patients, graft loss at 3 months occurred in 14 patients (11.9%). The optimal mode, called the GlycoTransplantTest, yielded an AUC of 0.95 for association with graft loss at 3 months. Using an optimised cutoff for this biomarker, sensitivity was 86% and specificity 89%. Negative predictive value was 98%. OR for graft loss at 3 months was 70.211 (p<0.001, 95% CI 10.876-453.231).

Conclusion: A serum glycomic signature is highly associated with graft loss at 3 months. It could support decision making in early retransplantation.

### **INTRODUCTION**

Since the first successful orthotopic liver transplantation (LT) by Starzl in 1963<sup>1</sup>, LT has become the treatment of choice for end stage liver disease and selected patients with hepatocellular carcinoma (HCC)<sup>2</sup>. Outcome after LT has steadily improved due to refinement of surgical techniques and introduction and improvement of immunosuppressive drugs. Survival rates now reach 96% and 71% at 1 and 10 years after LT respectively<sup>3</sup>. Graft loss occurs in 7 to 10% of adults<sup>4</sup> and requires retransplantation in these patients<sup>5</sup>, which can be early (caused by primary graft non-function or hepatic artery thrombosis) or late (ischemic cholangiopathy, chronic rejection or recurrence of the primary liver disease). Donor graft quality is increasingly recognized as a major driver of post-transplant outcome. Moreover, the shortage of donor organs has led to the increased use of extended criteria donors (ECD). These ECD grafts show unfavorable characteristics including advanced age, steatosis, DCD and others increasing the risk for ischaemia-reperfusion injury<sup>6</sup>.

The choice for retransplantation is based on a clinical appreciation by the transplant team and the use of liver enzymes and radiological imaging. However, it can be hard to define the need and the right timing for retransplantation, balancing between the wish to avoid a futile retransplantation and the need to perform an inevitable and life-saving retransplantation. Both pretransplant- and post-transplant clinical scores and biomarkers have been related to graft- and patient survival. A pretransplant evaluation using the Donor Risk Index (DRI)<sup>7</sup> identifies liver grafts at increased risk for graft failure based on donor criteria (age, donation after cardiac death, split/partial grafts, race, height and cause of death, cold ischemia time and allocation zone). Although DRI has not been challenged since its development more than 10 years ago, it lacks the individual prognostic value that would allow to discard inferior donor grafts from the donor pool<sup>8</sup>. A European donor risk index was developed using the Eurotransplant (ET) database resulting in the ET-DRI<sup>9</sup>. The major differences between both are the addition of latest

serum GGT and rescue allocation. Donor height and race were not included in this score. The predictive value of pre-operative MELD score remains unclear<sup>10-12</sup>.

Post-transplant markers can be divided in clinical scores and functional tests. The general concept is that these measure early allograft dysfunction (EAD) which has shown to be related to decreased organ and patient survival<sup>12, 13</sup>. The most widely accepted definition for EAD has been validated by Olthoff<sup>14</sup> and is based on postoperative laboratory values of bilirubin, INR and alanine or aspartate aminotransferases within the first 7 days after LT. Other scores are based on single measurement of (peak) AST or ALT values<sup>15</sup>, bilirubin<sup>12</sup>, lactate<sup>16</sup>, factor V<sup>17</sup> and platelet counts<sup>18</sup> but do not increase the diagnostic power of this definition. Functional tests include the indocyanine green (ICG)<sup>19, 20</sup> – plasma disappearance rate and the liver maximal function capacity (LiMax)<sup>21</sup>. These show encouraging results but lack a robust external validation. This overview points out that novel omics-based biomarkers have not been widely explored in this field.

We formerly showed that the analysis of the whole serum glycomic profile, which consists of measuring the N-glycans on the total protein content in serum (also called glycomics), does reflect hepatic (dys)function<sup>22, 23</sup>. Glycomic analysis of whole serum can be easily performed using a glycan analytical method that uses standard DNA-sequencing equipment<sup>24, 25</sup>. Based on this concept, we developed several biomarkers based on specific glycoalterations for the diagnosis of liver fibrosis<sup>26</sup> and cirrhosis<sup>25</sup>, HCC<sup>27, 28</sup> and NASH<sup>29-31</sup>. Based on the same technology a prognostic biomarker was defined that predicts the risk of HCC development in cirrhotic patients<sup>32</sup>. Recently we described that glycomic analysis of the perfusate before LT can identify patients at high risk to develop primary non function after LT<sup>33</sup>.

In this manuscript we studied the association between serum glycomics in the first week after transplantation and identified a serum glycomic signature associated with poor outcome at 3 months after LT.

#### MATERIALS AND METHODS

### Patients

A prospective study in the liver transplant unit of Ghent University Hospital (Belgium) was performed between 1 January 2011 and 28 February 2017. The cohort was split in a training set and a validation set for the analysis. Patients were included if a serum sample was available for analysis on day 7 after LT.

#### Design

Serum samples were collected on day 7 after LT. After centrifugation, serum samples were frozen to minus 21° Celcius. Clinical data were retrieved from the medical files. After collection of all serum samples, the serum samples were defrosted and glycomic analysis was performed. The resulting glycomic profiles were related to donor graft and patient survival.

### **Glycomic Analysis**

Five microliters of serum were processed according to the in-solution deglycosylation method described by Vanderschaeghe et al.<sup>26</sup>. Briefly, denaturing buffer containing SDS was added to the serum and incubated for 5 min at 95°C. Then, the samples were treated with Peptide N-glycosidase F to release the N-glycans from their denatured carrier proteins. After enzymatic removal of the terminal sialic acid residues, the glycans were labeled with 8-aminopyrene-1,3,6-trisulphonic acid and analysed using an ABI3130 DNA sequencer as described<sup>24</sup>. The result of this analysis is a total desialylated serum protein electropherogram (Fig. 1), which consists of 13 peaks. Each peak represents a well-identified glycan<sup>34</sup>. The numerical height of every peak is quantified and normalised to the sum of all peak heights, thus represented as a percentage of total peak height.

#### **Statistics**

The dataset was randomly split in a training set (70% of the data) and a test set (30% of the data)The serum glycomic signature contains 13 glycans. Using Lasso regression (R Package) an optimal model for graft loss at 3 months was selected based on cross-validated AUC on the training dataset. Based on the Youden index an optimal cut-off was defined. The model was validated on the test samples. In the complete sample cox regression analysis was performed and Kaplan Meier curves were derived based on the cut-off. Multivariate analysis was performed.

### **Ethics**

The study conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the Ghent University Hospital ethics committee. Informed consent was obtained in all patients.

### RESULTS

### **Patient Characteristics**

During the inclusion period, 281 patients received a LT. In 153 of these patients serum samples were prospectively collected. Finally 131 patients were included in the final analysis as the serum sample on postoperative day not available (n=16) or because the patient underwent retransplantation before day 7 due to primary non function (n=6). Seven liver transplants were retransplantations, 1 one was a second retransplantation.

Baseline characteristics are summarized in table 1. In this cohort, graft failure leading to retransplantation or patient death was observed in 14 patients (10.7%). The reason for graft failure were biliary complications (n=3), hepatic artery thrombosis (n=2), septic shock (n=3), small-for-size syndrome (n=3), extended primary graft failure (n=2) and rapidly progressive esophageal tumour (n=1). In this group, 3 patients died before retransplantation due to rapidly progressive esophageal tumour (n=1), septic shock due to ITBL (n=1) and septic shock in a patient with small-for-size syndrome (n=1).

#### Association Between Serum Glycomics And Graft Loss at 3 Months After LT

The study cohort was divided in a training set (n=91) and a validation set (n=40).

Lasso regression on training data set.

In this dataset of 91 patients, graft loss occurred in 9 patients. Glycomic analysis of serum samples on day 7 post transplantation in these patients results in data regarding the relative abundance of 13 glycans (Figure 1). The relative abundance of these glycans was compared between the patients with and without graft loss at 3 months after LT, and using Lasso regression an optimal model was fitted associated to graft loss. Different penalization parameters were applied. The Lasso regression model with the highest cross-validated AUC was selected. This model contained 11 predictors. The results of the Lasso regression can are in table 2.

The GlycoTransplantTest score was defined as the linear predictor (17.919 - 0.719\* RPeak1Serum + 3.600\* RPeak2Serum + ...) of this model. The resulting glycomics based biomarker was called the GlycoTransplantTest. The serum glycomic profile of these patients is predominantly characterized by increased undergalactosylation and an increased presence of fucosylated and triantennary glycans. Details are summarized in table 1. Receiver operating characteristic (ROC) curve analysis showed an area under the curve (AUC) of 0.95 (p<0.0001) for graft loss at 3 months (Figure 2). Using the Youden index, an optimal cut-off for the GlycoTransplantTest was defined at 1.76. When fitting a model with a more stringent penalization, only peaks 3, 5, 8 and 12 remained in the model. Since the obtained AUC on the training dataset was only 0.82, preference was given to the above model.

Validation of the Lasso regression model

In the test group, 40 patients remained of whom 5 experienced graft failure at 3 months. Here, the obtained AUC was 0.94 (p=0.0005).

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### Applying cut-off

Because of the low sample size of the test set, Using the cut-off of 1.76, the sensitivity for graft loss at 3 months was 86 % (95% CI : 0.60-0.96) and the specificity 89% (0.82-0.94). The positive predictive value (PPV) for graft loss in patient with a score above 1.76 was 50 % and the negative predictive value (NPV) 98% (Table 3).

Univariate and Multivariate analysis

The GlycoTransplantTest showed an excellent association with graft loss at 3 months after LT. Using logistic regression, several clinical donor and recipient parameters were studied for association with graft loss at 3 months, but only the GlycoTransplantTest showed an association with this outcome parameter (table 4). EAD and MEAF score were not associated with 3 months graft loss in this cohort.

This strong association was confirmed in a multivariate analysis, including the GlycoTransplantTest, the development of EAD and the DRI (OR 70.211, p<0.001, 95% CI 10.876-453.231). Correction for both EAD and MEAF score did not attenuate this strong association.

Survival analysis

Cox regression analysis showed a hazard ratio of 14.4 (95%CI: , 5.8-35.8) for graft loss (p<0.001) at 12 months. The discriminative factor for this glycomic biomarker is illustrated by the Kaplan Meier curve (Figure 3), where the majority of graft loss occurs in the first 3 months after LT.

## DISCUSSION

In this work we showed that the serum glycomic profile one week after LT is strongly associated with graft loss at 3 months after LT. Making use of Lasso regression an optimal model was fitted by incorporating information of 13 glycans which was called the GlycoTransplantTest. An optimal cut-off was defined at 1.76. Patients with a value below this threshold, showed a

strong association with graft loss at 3 and 12 months after LT. As can be appreciated from the Kaplan Meier curve (Figure 2), the optimal performance was observed for the prediction of graft loss at 3 months LT. It should be mentioned that patients experiencing PNF are not included in this analysis as they received retransplantation before day 7.

This work adds to the increasing evidence that glycomic-based biomarkers reflect in a reliable way specific dysfunctions occurring in the liver<sup>35</sup> and can be used as prognostic biomarkers<sup>32, 33</sup>. The major changes observed in patients with adverse outcome were increased undergalactosylation, and an increased presence of fucosylated and triantennary glycans. We and others formerly showed that the undergalactosylation in the whole serum N-glycome is caused by undergalactosylation of immunoglobulins and not by liver derived proteins<sup>26, 31, 36</sup>. The increased undergalactosylation is believed to be a reflection of the important inflammatory response in the failing liver due to factors related to ischemia/reperfusion damage<sup>37</sup>, infections or sepsis. Oweira et al. showed an independent association between postoperative inflammation after LT and graft loss and patient death<sup>38</sup>. This inflammatory response has been related to an increase of IL-6<sup>39</sup>, IL-2R, IL-7, IP-10, MIG<sup>37</sup>. Also IL-8, CCL2 and CCL5 are upregulated in the early postoperative phase resulting from the Nf-kB pathway<sup>37</sup>.

In contrast to the undergalactosylated glycans, the decrease of NA3, a triantennary glycan, is hepatocyte-driven<sup>26</sup> and could be caused by a disturbed glycosylation process in the failing liver. It is well known that glycosylation, one of the most important posttranslational modifications in human physiology, is strictly controlled by the upregulation of specific glycosyltransferases<sup>35</sup>. The action of N-acetylglucosaminyltransferase V (GnT-V), involved in the formation of precursor glycans of NA3, might be diminished in favor of an elevation of N-acetylglucosaminyltransferase III (GnT-III), responsible for the formation of bisecting GlcNAc structures, like NA2FB. Indeed, GnT-V competes for the same substrate as GnT-III<sup>27</sup>. As a matter of fact, NA2FB was shown to be significantly increased in patients with worse outcome.

Noteworthy, an increase of triantennary glycans (like NA3) can be considered a marker of liver regeneration. In human HCC samples an increased enzymatic activity of GnT-V has been observed<sup>40</sup>. Second, in a two-thirds partial hepatectomy model in rats, GnT-V activity was increased in hepatocytes and non-parenchymal cells during regeneration<sup>41</sup>. Possibly, the decreased levels of NA3 in patient with graft loss illustrate a lack of the required regeneration capacity after LTresulting in graft failure.

In a multivariate logistic regression model including the GlycoTransplanttest, EAD (as defined by Olthoff<sup>14</sup>), MEAF score<sup>42</sup> and DRI<sup>7</sup>, only the GlycoTransplantTest was an independent predictor of graft loss at 3 months after transplantation. According to these results this glycomic biomarker might be an attractive tool in the management of patients with suboptimal graft function in the first week after LT. In these patients it can be difficult to estimate whether the patient's liver function will recover or whether a retransplantation will be unavoidable. In this cohort we showed the best prognostic performance for graft survival with a single measurement at day 7 after LT. Hence, this marker could be an additional tool to assess the patients need for retransplantation.

It could also be an interesting tool in trials studying therapeutic strategies in early graft failure where liver grafts at increased risk of failure could be identified.

A limitation is the monocentric character of this study and the current absence of external validation. However, it is a prospective study where a training and validation cohort was used and crossvalidation was applied. To our knowledge however, this is the first biomarker that offers this high predictive value with an odds ratio OR for graft loss at 3 months of more than 70 using the cut-off applied in this study.

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The GlycoTransplantTest is measured using a routine DNA-sequencer and can be implemented on a commercial capillary electrophoresis platform (V8 Analyzer, Helena Biosciences Ltd, UK) that is available in a routine clinical lab environment, which will make the technology widely accessible.

In line with previous reports of our group these data highlight the value and potential of glycomics-based biomarkers in liver disease. A glycomic assessment of serum at day 7 after LToffers a reliable marker of graft function that is independently associated with graft survival within the first 3 months after LT. Hence, it could be an additional tool for guidance in decision making when a retransplantation is considered. Furthermore, it could be integrated in clinical trials as a surrogate marker of graft surival. The application of this technology on high-throughput microfluidics CE platforms could facilitate an easy implementation in clinical practice.

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#### **Figure Legends**

### Figure 1: The glycomic analysis and GlycoTransplantTest

The structures of the N-glycan peaks in the total serum of a cirrhotic patient 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 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 NA2F, peak 8 is the triantennary glycan NA3, peak 9 is the branching alpha-1,3-fucosylated triantennary glycan NA3Fb, peak 9 is the core-alpha-1,6-fucosylated triantennary glycan NA3Fb, peak 10 is the branching alpha-1,3-fucosylated and core alpha-1,6-fucosylated triantennary glycan NA3Fbc, peak 11 is a tetra-antennary (NA4) and peak 12 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.

Figure 2: Receiver operating characteristic (ROC) curve analysis of the GlycoTransplantTest showed an area under the curve (AUC) of 0.95 (p<0.0001) for graft loss at 3 months. Figure 3. Kaplan Meier curve shows a significant better survival of the liver graft in patients with a value of the GlycoTransplantTest above 1.76. Legend: Full line: Pos >1.76, Dashed Line: Neg <=1.76. Day 0 is the day of the GlycoTransplantTest, which is day 7 after LT. The supplementary table associated with this article is available at http://links.lww.com/TP/C66

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	Overall N= 131	Graft survival n- 117	Graft failure n-14	p value			
Recipient characteristics							
Sex (male %)	59%	58%	44%	0.218			
Age : median	56	55	59 (17.629)	0.794			
(SD),y	(12.77)	(12.99)					
Underlying liver							
disease							
Viral hepatitis	11%	13%	22%	0.583			
Alcohol liver	35%	38%	34%	0.623			
disease							
PBC/PSC/AIH	12%	11%	13%	0.343			
NASH	15%	15%	14%	0.683			
HCC,yes(%)	13%	3%	0%				
other	14%	20%	17/	0.587			
MELD: median	18	16	24 (10.41)	0.567			
(SD)	(10.25)	(10.74)					
Donor characte	ristics			-			
Age : median	54	56	36 (18.3)	0.961			
(SD),y	(17.92)	(17.48)					
Donor Risk	1,78	1.76	1.60 (0.49)	0.696			
Index	(0.41)	(0.37)					
NHBD, yes (%)	13%	7.7%	64%	0.065			
Surgical charac	teristics						
Cold ischemia	349	421	338 (251)	0.293			
time : median	(149)	(144)					
(IQR) min		0.0	40 (40 04)	0.004			
vvarm ischemia	36	36	49 (18.01)	0.684			
time: median	(13.7)	(14.18)					
(IQK) MIN							
Postoperative							
course							

 Table 1: Baseline characteristics of patients.

\*According to Olthoff

ALT: alanine aminotransferase, AST: aspartate aminotransferase, HCC: hepatocellular carcinoma, IQR: interquartile range, MELD: Model for End-Stage Liver Disease; NHBD: non heart beating donor, POD: postoperative day, PBC: primary biliary cholangitis, PSC: primary sclerosing cholangitis, AIH: autoimmune hepatitis– *Independent Student T test*  **Table 2**. Overview of serum glycans included in the model using Lasso Regression. The coefficient and Odds Ratio's (OR) are reported for every glycan. Legend. NGA2F is an agalacto, core-alpha-1,6-fucosylated biantennary glycan, NGA2FB is an agalacto, core-alpha-1,6-fucosylated bisecting biantennary, NG1A2F are 2 isomers of single agalacto, core-alpha-1,6-fucosylated biantennary structures, NA2 is a bigalacto biantennary glycan, NA2F is a bigalacto, core-alpha-1,6-fucosylated bisecting biantennary glycan, NA2FB is a bigalacto, core-alpha-1,6-fucosylated bisecting biantennary glycan, NA2FB is a bigalacto, core-alpha-1,6-fucosylated bisecting biantennary glycan, NA3FB is a triantennary glycan, NA3FB is a branching alpha-1,3-fucosylated triantennary glycan, NA3Fbc is a branching alpha-1,3-fucosylated and core alpha-1,6-fucosylated triantennary glycan NA3Fbc and NA4Fb is a branching alpha-1,3-fucosylated tetra-antennary (NA4Fb) glycan. These figures are also represented in figure 1.

Serum glycan	Coefficient	OR
NGA2F	-0.719	0.49
NGA2FB	3.600	36.58
NG1A2F	-1.077	0.34
NG1A2F	0.243	1.27
NA2	-0.350	0.70
NA2F	0.157	1.17
NA2FB	-0.552	0.58
NA3	0.716	2.05
NA3FB	0.440	1.55
NA3FBC	-0.698	0.50
NA4FB	-1.387	0.25

**Table 3**. Predictive value of the glycomic biomarker for graft loss at 3 months after liver transplantation if value above 1.76. PPV: positive predictive value. NPV: negative predictive value

		95% Confidence		
Sensitivity	0.86	0.60-0.96		
Specificity	0.89	0.82-0.94		
PPV	0.50	0.31-0.69		
NPV	0.98	0.93-0.99		

**Table 4**. Association between clinical parameters and graft loss at 3 months, univariateand multivariate analysis (Logistic Regression)

	Univariate Analysis			Multivariate Analysis		
	Odds	95% CI	p value	Odds	95% CI	p value
	Ratio			Ratio		
MELD score	1.040	0.975-	0.225			
recipient		1.109				
Donor Length	1.027	0.938-	0.565			
		1.124				
Donor Weight	1.067	0.998-	0.058			
		1.141				
Donor Age	1.005	0.954-	0.859			
		1.058				
Donor Sex	0.574	0.143-	0.433			
		2.301				
Cold Ischemia Time	0.998	0.994-	0.465			
		1.003				
Warm ischemia time	1	0.989-	0.982			
		1.011				
Donor Risk Index	0.755	0.130-	0.753			
		4.367				
EAD	0.296	0.069-	0.102	2.54		0.13
		1.274				
MEAF score	0.33	0.093-	0.099	0.68		0.58
		1.22				
GlycoTransplantTest	4.004	1.968-	<0.001	4.119	1.949-	<0.001
(continous scale)		8.144			8.704	
GlycoTransplantTest	49.0	9.770-	<0.001	70.211	10.876-	<0.001
(according to cut off		245.752			453.231	
1.76)						





Time

Figure 2





