The potential of glycomics as prognostic biomarkers in liver disease and liver transplantation

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Voor mijn ouders en mijn zus, Maud.
Voor jullie steun bij de start van dit avontuur.

Voor mijn echtgenote Sofie en zoon Henri.
Voor jullie steun om het tot een goed einde te brengen.

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ABBREVIATIONS

ACR: Acute cellular rejection

AIH : auto immune hepatitis

AFP: alpha phoeto protein

AJCC: American Joint Committee on Cancer staging system

ALF: acute liver failure

ALT : alanine aminotransferase

AST : aspartate aminotransferase

APRI : AST to Platelet Ratio Index calculator

ARFI: acoustic radiation force impulse imaging

BCLC: Barcelona Clinic Liver Cancer (staging system)

cACLD: compensated advanced chronic liver disease'

CE: ceruloplasmin

CMV: cytomegalovirus

CNI: calcineurin inhibitor

CSF1R : colony stimulating factor 1 receptor

DAA: direct acting antiviral

DBD: Donors after brain-dead

DCD: donors after circulatory death

DRI: donor risk index

DSA: donor-specific antibodies

DSA FACE: DNA sequencer-assisted fluorophore-assisted carbohydrate electrophoresis

EAD : early allograft dysfunction

ECD: extended criteria donors

ER: endoplasmic reticulum

ERAD: ER-associated protein degradation

ERCP: endoscopic retrograde cholangiopancreaticography

ET: Eurotransplant

FLISA : Fluorophore-linked immunosorbent assay

Gal: galactose

GGT: Gamma-glutamyltransferase

Glc : glucose

GlcNAc : N-acetyl-D-glucosamine

GDP : guanosine diphosphate

HBV: hepatitis B virus infection

HC: healthy controls

HCC: hepatocellular carcinoma

HCV: hepatitis C virus infection

HGF: Hepatocyte Growth factor

HLE-EPI: Epirubicin resistant human hepatocellular carcinoma cell lines

HLE-MIT: Mitoxantrone resistant human hepatocellular carcinoma cell lines

HRG: histidine-rich glycoprotein

HRS: hepatorenal syndrome

IGOT: isotope-coded glycosylation site–specific tagging

IMS: Ion mobility spectrometry

INF: interpheron

INR: International Normalized Ratio

ITBL: ischemic type bile duct lesions

LC-ESI-HCD-MS/MS: Liquid chromatography – Electrospray Ionisation - Higher Energy Collision Dissociation tandem mass spectometry

LT: liver transplantation

MALDI-QIT-TOF : Matrix Assisted Laser Desorption/Ionization, Quadrupole Ion Trap Time of Flight

Man: mannose

MELD: Model for End-Stage Liver Disease

MMF: mycophenolate mophetil

MS : Mass spectrometry

m-TOR: mammalian target of rapamycin

NAFLD : non alcoholic fatty liver disease

NASH : non alcoholic steatohepatitis

PBC: primary biliary cholangitis

PNF: primary non function

PSC: primary sclerosing cholangitis

pSWE: point shear wave elastography

PT: prothrombin time

PVT: portal vein thrombosis

SELDI : Surface-enhanced laser desorption/ionization

TAE: Transarterial embolisation

TE: transient elastography

TIPS : transjugular intrahepatic portosystemic shunt

TMT : tandem mass tag

TNF: tumor necrosis factor

SBP : spontaneous bacterial peritonitis

UGGT: UDP-glucose:glycoprotein glucosyltransferase

I.   Introduction

I. INTRODUCTION

1.1 GENERAL INTRODUCTION

The most advanced stage of chronic liver disease is called cirrhosis[1] (Figure 1). The presence of cirrhosis can lead to major complications linked to the development of portal hypertension and hepatocellular carcinoma. The WHO reports that liver cirrhosis accounts for 1.8% of all deaths in Europe (170,000 deaths per year) with the highest rates observed in south-eastern and north-eastern Europe[2]. The ultimate therapy for these complications is liver transplantation[3].
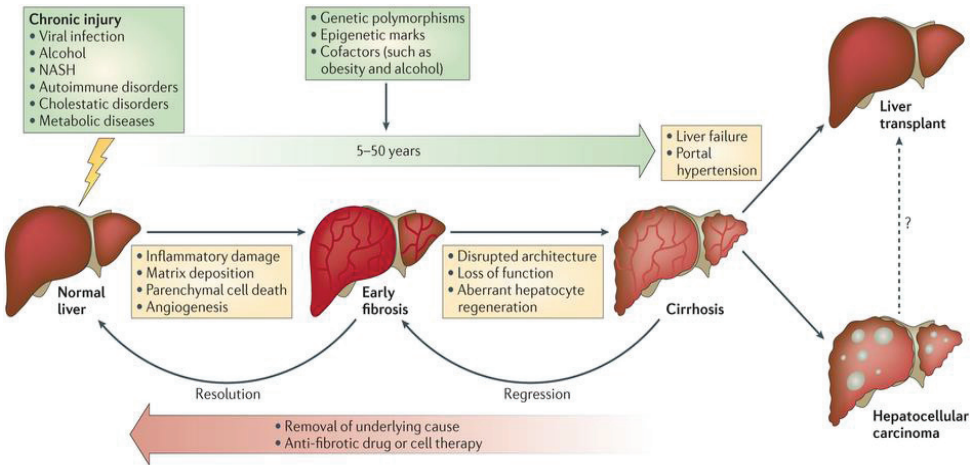
Figure 1. Evolution of chronic liver disease. *(adapted from Pellicoro et al. Nature Reviews Immunology 2014;14:181-194).*

Cirrhosis is the result of increasing hepatic fibrosis, caused by chronic liver injury. Etiologies include viral infection (eg. chronic hepatitis B and C), alcohol, non-alcoholic steatohepatitis (NASH), autoimmune liver disease, excessive iron or copper accumulation amongst others. Hepatic fibrosis is the wound-healing response of the liver. The iterative injury causes inflammatory damage; subsequent matrix deposition, parenchymal cell death and angiogenesis lead to progressive fibrosis deposition. Although scarring accumulates very slowly (eg. in chronic hepatitis C median time to cirrhosis is more than 30 years), once cirrhosis is established, the potential for a reversal of this

process is decreased and complications occur. Genetic polymorphisms, epigenetic marks and cofactors (such as obesity and alcohol) can modulate the risk of fibrosis progression[4].

Hepatocarcinogenesis is a multi-step process in which hepatic precancerous lesions progress into early HCC[5]. These precancerous lesions, macroscopic dysplastic nodules, result from cirrhosis. Dysplastic nodules can be divided into low-grade dysplastic nodules and high-grade dysplastic nodules, the latter carrying a higher risk of malignant transformation[6].

Various etiological factors, particularly inflammation and viral hepatitis, appear to contribute significantly to approximately 90% of HCC cases by creating phenotypically altered hepatocytes. The stepwise progression from altered hepatocytes to dysplastic nodules, or precancerous lesion, occurs as a consequence of chronic inflammation and genomic alterations, which commonly precede HCC[7–9]. The accumulation of genetic and epigenetic changes, such as the loss of tumor suppressor genes and the gain of an oncogene, causes the development of primary tumor cells[10]. In the liver, cytokines and reactive oxygen and nitrogen species produced by inflammatory cells have been shown to mediate liver damage and induce the liver's regenerative response[10]. This predisposes the proliferating cell to a variety of genetic changes at the genomic and transcriptional levels.

Typical findings in HCC tumors are loss or mutation of tumor suppressor genes such as TP53[11], retinoblastoma RB1[12], CDKN2A (p16INK4A)[13] and insulin-like growth factor-2 receptor[14], which are strongly associated with carcinogenetic signaling pathways. Gain of function mutations have also been observed in HCC, for example mutations in CTNNBI (β-catenin)[15], which results in the deregulation of similar signaling pathways in HCC[15].

Recently, a new model for HCC carcinogenesis has been proposed, based on the hypothesis that HCC could be derived from progenitor cells or de-differentiated transformed cells. This could explain the heterogeneity of HCC morphology, behavior and molecular profiles[10,16]. These bi-potential progenitor cells can give rise to hepatocytes or cholangiocytes, which could possibly develop into HCC or intrahepatic cholangiocarcinoma, respectively[17,18].

In this work we explore the **prognostic potential of glycomics-based biomarkers for outcome** in patients with **cirrhosis** and for patients who undergo **liver transplantation**.

This introduction will cover (1) a general introduction of cirrhosis and its major complications, (2) a general introduction on liver transplantation including a review of non invasive biomarkers for the diagnosis of acute cellular rejection and (3) an introduction in glycosylation and glycomics with a review of glycomics-based biomarkers in liver disease.

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1.2 CIRRHOSIS. DIAGNOSIS, COMPLICATIONS AND TREATMENT

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Cirrhosis: Reviewing the literature and future perspectives

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1.2.1 ABSTRACT

Cirrhosis is the final stage of chronic liver disease and has many causes, including viral hepatitis, excessive alcohol intake, and non-alcoholic steatohepatitis. When decompensated cirrhosis develops, complications occur that affect quality of life and patient survival. Cirrhosis has a large burden of disease and is responsible for almost 2% of deaths in Europe. Cirrhotic patients are in need of early diagnosis and a careful follow-up for the prevention and detection of complications. The ultimate treatment for end-stage cirrhosis is liver transplantation. This review will cover clinical aspects of cirrhosis and uncover future trends in the care of these patients.

1.2.2 INTRODUCTION

Cirrhosis is the final stage of chronic liver disease. It results in distortion of the hepatic architecture by fibrosis, and the formation of regenerative nodules[1]. It is the result of progressive liver fibrosis caused by chronic liver diseases, including viral hepatitis, alcoholic liver disease, NASH, autoimmune liver disease, and genetic disorders, amongst others. Recent reports support the finding that the early stages of cirrhosis are reversible on a microscopic level with adequate treatment of the underlying liver disease[2]. However, at more advanced stages, cirrhosis is considered irreversible. Cirrhosis is the source of a variety of complications, which result in a reduction in the life expectancy of these patients. At this stage, liver transplantation is the only curative treatment option[4].

1.2.3 EPIDEMIOLOGY AND ETIOLOGY

Cirrhosis has a large burden of disease. It is the eighth leading cause of death and is responsible for 1.2% of all deaths in the USA[5]. According to the Global Burden of Disease study, the worldwide prevalence of cirrhosis is increasing[6]. In the USA, the most common causes of cirrhosis are chronic hepatitis C virus (HCV), alcoholic liver disease, and non-alcoholic liver disease[7]. In Europe, liver cirrhosis accounts for 1.8% of all deaths, amounting to 170,000 deaths per year[3]. Worryingly, the reported incidence of cirrhosis remains stable or is increasing in several countries, including both the UK[8] and Ireland[3]. In Europe, the main causes are alcoholic liver disease, NASH, and HCV[3]. The four most frequent causes of cirrhosis worldwide are chronic hepatitis B virus (HBV) and HCV, alcoholic liver disease, non-alcoholic fatty liver disease (NAFLD), and haemochromatosis. A variety of other diseases can result in cirrhosis, although these are less frequent[9].

Alcohol

Excessive alcohol intake remains the number one cause of cirrhosis in Western countries. A daily intake of ≥60 g/day for men, and ≥40 g/day for women is considered harmful. Chronic intake of alcohol can also accelerate the natural progression of chronic HBV or HCV[10], and haemochromatosis. Alcohol abstinence is the cornerstone of treatment and can reverse the disease course[11].

Viral Hepatitis

Chronic HBV and HCV are leading causes of cirrhosis, especially in endemic regions like South East Asia and Sub-Saharan Africa. According to the disease stage, finite treatment with pegylated interferon or long-term therapy with nucleos(t)ide analogues is appropriate in HBV patients[12]. The introduction of interferon-free treatment for HCV has been important, as it has resulted in improved treatment response without significant side effects[13]. However, access to these new direct-acting agents remains a challenge due to high costs. Hepatitis A and E do not develop into chronic hepatitis in immunocompetent patients and are not considered risk factors for cirrhosis.

Non-Alcoholic Fatty Liver Disease

NAFLD is related to the presence of metabolic syndrome in association with obesity, diabetes, and/or arterial hypertension. A subset of these patients will develop signs of NASH, which can lead to the development of fibrosis and subsequently cirrhosis[14,15]. It is an increasing health problem, especially in the Western world[6]. Treatment is based on dietary measures and exercise[14].

Haemochromatosis

Hereditary haemochromatosis is an autosomal recessive disorder characterised by excessive intestinal absorption of dietary iron, which results in a pathological increase in total body iron stores[16]. End-organ liver damage can occur, in turn leading to cirrhosis. Phlebotomy has been indicated to remove excessive iron stores[17].

Autoimmune Hepatitis

Autoimmune hepatitis is a rare disease affecting 16–18 cases per 100,000 inhabitants in Europe. More than 30% of adult patients and ~50% of children have cirrhosis at diagnosis, due to an insidious

disease course[18]. Treatment is based on immunosuppressive agents including corticosteroids and azathioprine[18].

Primary Biliary Cholangitis and Primary Sclerosing Cholangitis

Primary biliary cholangitis (PBC) and primary sclerosing cholangitis (PSC) are autoimmune diseases that affect the small and the large bile ducts, respectively. PBC can lead to progressive fibrosis resulting in cirrhosis. In PSC patients, prolonged extrahepatic cholestasis can induce the development of portal fibrosis leading to cirrhosis[19]. Ursodeoxycholic acid can slow down disease progression in PBC and can be used in PSC[20]. In PBC, newer agents, like obeticholic acid, are promising treatment options[21].

Rare Causes of Cirrhosis

Other causes of cirrhosis include a reaction to drugs, Budd-Chiari syndrome, Wilson's disease, alpha-1 antitrypsin deficiency, granulomatous liver diseases, right-sided heart failure, and veno-occlusive disease amongst others[9]. A specific aetiology can be determined in 85–90% of patients[22].

1.2.4 CLINICAL MANIFESTATIONS

Cirrhosis can be compensated without overt complications, or decompensated with the appearance of complications. The three major complications of cirrhosis are the consequences of portal hypertension (e.g. ascites, variceal bleeding, etc.), hepatocellular insufficiency (e.g. icterus), or the appearance of hepatocellular carcinoma (HCC).

Patients with compensated cirrhosis may present with nonspecific symptoms or may even be asymptomatic. They can complain of anorexia, weight loss, or fatigue. When decompensation develops, patients may present with jaundice, pruritus, signs of upper gastrointestinal bleeding, abdominal distension due to ascites, or confusions due to hepatic encephalopathy[23]. Hypogonadism may occur in men, which can manifest as impotence, infertility, or loss of libido[24]. In women, amenorrhoea or irregular menstrual bleeding are common[25]. Typical signs at clinical examination include jaundice, stellate angiomas, palmar erythema, foetor hepaticus, asterixis, signs of hypogonadism, and feminisation in males. Other signs include indicators of portal hypertension such as ascites, cutaneous collateral venous circulation, and splenomegaly[23].

1.2.5 DIAGNOSIS

Laboratory Findings

Laboratory abnormalities may be the first indication of liver cirrhosis. Though bilirubin levels may be normal in compensated cirrhosis, the levels rise as cirrhosis progresses. Levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) are moderately elevated in cirrhosis; however, normal aminotransferase levels do not exclude cirrhosis. Alkaline phosphatase is usually mildly elevated in cirrhosis. Levels higher than 2 or 3-times the upper limit of normal suggest an underlying cholestatic liver disease, such as PSC or PBC[20]. Gamma-glutamyl transpeptidase levels correlate well with alkaline phosphatase, but are more elevated in alcohol induced chronic liver disease[26].

Once the synthetic function of the liver is affected, albumin levels decrease and prothrombin time levels increase as key proteins involved in the coagulation cascade are produced in hepatocytes. Low platelets can appear in the case of hypersplenism[27].

Imaging

Ultrasonography is the first step in liver imaging. It is non-invasive, widely available, affordable, and well accepted by patients. Liver volume can be normal, enlarged, or diminished, especially in advanced cirrhosis[28]. Often a nodular deformation of the liver can be observed. Other typical signs include atrophy of the right lobe of the liver, and hypertrophy of the caudate or left lobes.

When portal hypertension develops, Doppler imaging can reveal an enlarged portal vein, enlarged collateral veins, and decreased portal flow[29]. Ultrasonography is useful for the detection of hepatic nodules and is the backbone of screening programmes for the early detection of HCC[30,31]. Detection of hepatic nodules demands further characterisation using computed tomography or magnetic resonance imaging.

Non-Invasive Markers of Cirrhosis

Hepatologists are increasingly adopting the use of non-invasive markers of fibrosis and cirrhosis. These include biological markers and transient elastography (TE). Liver fibrosis can be staged using 1-dimensional ultrasound (FibroScan®, Echosens, France) [32], which measures the velocity of a low-frequency (50 Hz) elastic shear wave propagating through the liver. The stiffer the tissue, the faster the shear wave propagates. Ultrasound elastography can currently be performed based on two physical principles: strain displacement/ imaging, and shear wave imaging and quantification[33]. The

latter includes point shear wave elastography (pSWE), also known as acoustic radiation force impulse imaging (ARFI; Virtual touch tissue quantification™, Siemens Healthcare; elastography point quantification, ElastPQ™, Philips) and 2D-shear wave elastography (2D-SWE; Aixplorer™ Supersonic Imagine, France). A major advantage of pSWE/ARFI is that it can be easily implemented on modified commercial ultrasound machines (Acuson 2000/3000 Virtual Touch™ tissue quantification, Siemens Healthcare, Germany; ElastPQ, iU22xMATRIX, Philips, Netherlands). This results in a combined approach of conventional ultrasonography with TE[34]. Correct interpretation of pSWE/ARFI results should systematically take into account potentially confounding parameters: fasting for at least 2 hours, levels of transaminases (<5-times the upper limit of normal), absence of extrahepatic cholestasis, and absence of right heart failure[33]. According to the European Association for the Study of the Liver (EASL) clinical practice guidelines, TE is a reliable method for the diagnosis of cirrhosis in patients with chronic liver diseases. TE is generally better at ruling-out rather than suggesting cirrhosis and has a negative predictive value >90%[34].

FibroTest©, a patented biomarker (combining six serum markers with the age and gender of the patient: alpha-2-macroglobulin, haptoglobin, apolipoprotein A1, gamma-glutamyl transpeptidase, total bilirubin, and ALT), and APRI (AST to Platelet Ratio Index calculator) are the most widely used and validated biological markers[35]. Fibrotest and APRI show an area under a receiver operating characteristic curve (AUROC) of 0.86 and 0.84[36], respectively, for the diagnosis of cirrhosis.

Liver Biopsy

The gold standard for the diagnosis of cirrhosis is a histological examination. However, this should not be performed in all cirrhotic patients. A biopsy should be considered in patients in whom the diagnosis is in question, and when knowledge of a specific diagnosis is likely to alter the management of the disease[37]. A liver biopsy can be performed percutaneously, transjugularly, or laparoscopically. There is an inherent risk of bleeding, and severe bleeding occurs in between 1 in 2,500 and 1 in 10,000 biopsies performed using an intercostal percutaneous approach[37].

1.2.6 MAJOR COMPLICATIONS

Cirrhotic patients are at risk for the development of complications, therefore cirrhotic patients should be observed more closely for decompensated cirrhosis. Once decompensation develops, the patient should be considered for liver transplantation[4]. Many complications of cirrhosis develop as a result of portal hypertension, an increased pressure in the portal circulation defined as an elevation

of the hepatic venous pressure gradient to >5 mmHg[38]. The haemodynamic abnormalities associated with portal hypertension cause the most severe complications of cirrhosis, including ascites, hepatic encephalopathy, and bleeding from gastro-oesophageal varices. Ascites is the accumulation of fluid in the peritoneal cavity. It is treated with diuretics and sodium restriction. Some patients require repeated therapeutic paracentesis, or transjugular intrahepatic portosystemic shunt (TIPS) placement[39]. In patients with ascites, spontaneous bacterial peritonitis (SBP) may occur. Patients may be asymptomatic, present with altered mental status, or be seriously ill with a high fever, abdominal tenderness, and pain. The diagnosis is established by an elevated ascitic fluid absolute polymorphonuclear leukocyte count (≥250 cells/mm^3). The mortality is high if prompt antibiotic treatment and albumin substitution are not initiated[39].

Hepatorenal syndrome (HRS) can develop in patients with advanced cirrhosis. HRS is the development of renal failure in patients with advanced chronic liver disease who have portal hypertension and ascites[40]. Around 40% of these patients will develop HRS during the natural history of their disease. It is caused by vasoconstriction of the renal circulation and intense systemic arteriolar vasodilatation, which results in reduced systemic vascular resistance and arterial hypotension. Following liver transplantation, the histological appearance of the kidneys is normal and the kidneys often resume normal function[39]. Treatment of HRS is based on the treatment of the precipitating factors; adequate volume replacement with albumin and vasoconstriction therapy with vasopressin analogues, such as terlipressin[39].

Variceal haemorrhage is a dramatic event that typically presents as haematemesis and/or melaena. The mortality rate is high (20%, 30-day mortality) and treatment requires a multidisciplinary approach[41] including antibiotic treatment and endoscopic haemostasis. In selected patients, early TIPS placement can increase survival[42].

A typical complication of cirrhosis is the occurrence of portal vein thrombosis (PVT). According to a large prospective trial in France, the 5-year cumulative incidence of PVT was 10.7%[41]. PVT is associated with the severity of liver disease at baseline and anticoagulation is indicated in patients waiting for liver transplantation[43].

Hepatic encephalopathy encompasses a spectrum of potentially reversible neuropsychiatric abnormalities including confusion, altered level of consciousness, and coma[44]. Signs can easily be overlooked when they are limited to psychomotor slowing, a lack of attention, or sleep disturbances. Hepatic encephalopathy can be scored using the West Haven criteria. A typical sign of encephalopathy is the presence of asterixis. Treatment is based upon addressing the precipitating factors using synthetic disaccharides (e.g. lactulose) and nonabsorbable antibiotics (e.g. rifaximin)[44].

Liver cirrhosis is the most important risk factor for the development of HCC. HCC represents up to 85% of the primary liver cancer burden[45]. In patients with compensated cirrhosis the annual incidence of HCC ranges from 1–8%[46]. It is mandatory for an ultrasonography to be taken every 6 months to ensure early detection of HCC[47-48].

1.2.7 FOLLOW UP, PREVENTION OF COMPLICATIONS AND TREATMENT

The natural course of cirrhosis is variable and can be well tolerated for many years. In these patients the primary goal should be to prevent the occurrence of complications. Slowing or even reversing the progression of liver disease can be achieved by addressing the underlying liver disease. Abstinence from alcohol improves survival in alcoholic cirrhosis[49]. Achieving a sustained viral response in HCV with antiviral treatment lowers liver-related mortality[50].

The presence of impaired hepatic metabolism and renal excretion denotes a need for caution with many medications, which may subsequently necessitate dose adjustments or should even be avoided[49,51]. Nephrotoxic agents can precipitate HRS and should be used cautiously. Careful monitoring for the development of complications and, if possible, the prevention of complications, is the cornerstone of the treatment of a cirrhotic patient. Cirrhotic patients should undergo screening for oesophageal varices with upper endoscopy. However, according to the recent Baveno VI guidelines[52], patients with a liver stiffness <20 kPa, and a platelet count >150,000 can avoid screening. Patients with medium or large varices require primary prevention with non-selective beta blockers or endoscopic band ligation. The role of carvedilol remains unclear[52]. Furthermore, platelet levels <100,000 can increase risk for surgery.

In a study, it was demonstrated that in patients with ascitic fluid protein <15 g/L and without prior SBP, norfloxacin (400 mg/day) reduces the risk of SBP and improves survival. In these patients, long-term primary prophylaxis should be considered[41]. Empirical antibiotics should be started immediately following the diagnosis of SBP. Furthermore, albumin (1.5 g/kg at diagnosis and 1 g/kg on Day 3) should be administered in order to decrease the risk of HRS[41].

The presence of hepatic encephalopathy can be extremely subtle. Precipitating factors including dehydration, infection, and variceal bleeding should be avoided or addressed as soon as possible. The ultimate treatment for cirrhosis is liver transplantation, and excellent long-term results have been demonstrated[53]. It should be considered in patients with decompensated cirrhosis. The final decision depends upon the severity of the liver disease and the absence of contraindications[4].

Patients who develop HCC should be managed according to the Barcelona Clinic Liver Cancer (BCLC) staging system[31]. Single HCC lesions in Child–Pugh A patients are eligible for resection or ablation. Intermediate stage disease patients are offered locoregional therapy including transarterial chemoembolisation or radioembolisation. In advanced or metastatic disease, sorafenib is the only remaining option; it improves median overall survival from 6 to 9 months. In patients with lesions that meet the 'Milan criteria' liver transplantation should be considered[31].

1.2.8 PROGNOSIS

The prognosis of patients with compensated cirrhosis is excellent. Transition from the compensated to the decompensated stage occurs at a rate of 5–7% per year[11]. The median survival rate in compensated cirrhosis is >12 years[11]. Once patients develop complications of cirrhosis, such as ascites, variceal bleeding, or HRS, they are considered to have decompensated cirrhosis and their prognosis is worse.

Two models are commonly used for prognosis evaluation: the Child–Pugh classification and the Model for End-Stage Liver Disease (MELD). The Child–Pugh classification includes the variables serum albumin and bilirubin, ascites, encephalopathy, and prothrombin time[54]. The ranges from 5 to 15, and patients are divided into Child–Pugh A (score 5–6), B (score 7–9), or C (score 10–15). One-year survival rates for Child– Pugh A, B, and C patients are 100%, 80%, and 45%, respectively[55]. MELD score is calculated using bilirubin levels, creatinine, and international normalised ratio[56]. It is now used for prioritising patients on the liver transplant waiting list. Patients with a MELD score of >10 should be referred to a liver transplant centre for evaluation.

There is a growing interest in the use of non-invasive tests for the prognosis of chronic liver disease, particularly for TE in patients with cirrhosis[34]. The Baveno VI consensus paper[52] introduced the term 'compensated advanced chronic liver disease' (cACLD). This term applies to patients with chronic liver disease at increased risk of developing clinically significant portal hypertension, defined as a hepatovenous pressure gradient of ≥10 mmHg. TE values <10 kPa in the absence of other known clinical signs rule out cACLD. Values between 10–15 kPa are suggestive of cACLD but need confirmation. Values >15 kPa are highly suggestive of cACLD. Patients with cACLD are at an increased risk for complications and should be referred to a liver disease specialist[52].

1.2.9 CONCLUSION

Cirrhosis is the final stage of chronic liver disease. The aim of a clinician dealing with cirrhosis should be to prevent the development of major complications. A new trend in this field is the adoption of non-invasive techniques, e.g. TE for diagnosis of cirrhosis and follow-up of cirrhotic patients, as they are an emerging tool for risk stratification. In cirrhotic patients the performance of an ultrasonograph every 6 months remains of utmost importance for early detection of HCC. Decompensated patients have a dismal prognosis and should be referred to a specialised hepatological centre, as liver transplantation should be considered in these patients.

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1.3 LIVER TRANSPLANTATION

1.3.1 GENERAL INTRODUCTION

The first liver transplantation was performed by Thomas Starzl in the United States in 1963[1]. After a challenging start, liver transplantation has become the standard of care for acute and chronic liver failure and selected patients with hepatocellular carcinoma with more than 80 000 liver transplantations performed to date[2]. The breakthrough of liver transplantation has been possible after the introduction of immunosuppressive agents like cyclosporine[3] and in the improvement of surgical techniques and peri-operative intensive care[4]. In the eighties and the nineties the number of liver transplantations showed an exponential growth which has stabilized since the start of this century[2] (Figure 1). Nowadays a major challenge in liver transplantation, which hampers its further expansion, is the shortage of donor organs.
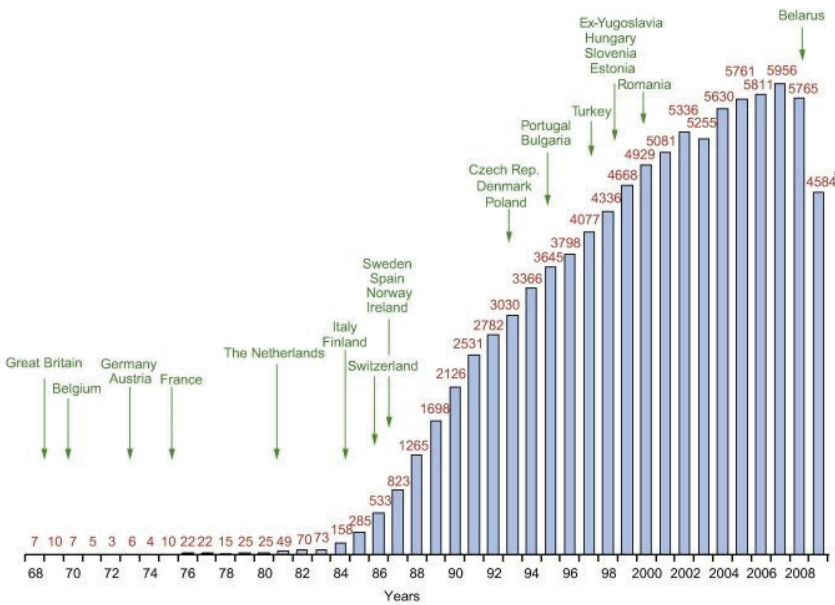
European Liver Transplant Registry

Figure 1. Evolution of 93,634 LTs performed in Europe since May 1968. Arrows indicate the year the first LT was performed in indicated countries. *This decrease is owed to the fact that some centers did not yet send their updating further to the recent changes of the questionnaire.Source: Adam et al.*[2]

1.3.1.1 Indications for liver transplantation

Liver transplantation is indicated in patients with end-stage liver disease, hepatocellular carcinoma (selected patients) and acute liver failure (Figure 2).

The most common indication is decompensated cirrhosis with the occurrence of major complications such as variceal haemorrhage, ascites, encephalopathy etc[5]. Liver transplantation can be considered in these patients if liver transplantation would extend the life expectancy beyond what can be expected from the natural history of the underlying liver disease or if it is likely to improve the quality of life. The major causes of cirrhosis are chronic alcohol intake, chronic hepatitis C and B and non-alcoholic fatty liver disease. The primary cause of cirrhosis should be treated if possible. This includes abstinence of alcohol for at least 6 months and appropriate treatment of HBV and HCV patients with antiviral drugs[5]. Other causes of end-stage liver disease are cholestatic liver diseases, eg. primary biliary cholangitis or primary sclerosing cholangitis and metabolic diseases[2].

Acute liver failure develops in healthy, often young patients without underlying liver disease and can be caused by toxic agents (eg. paracetamol) or viruses (hepatitis A or B)[6]. These patients show a rapid deterioration of their general status with the development of encephalopathy. Some of these patients require urgent liver transplantation, within 48 hours after the development of the clinical syndrome. Liver transplantation has revolutionized the prognosis of these patients causing survival to increase from 10-20% to 78-80% at 1 year[7].
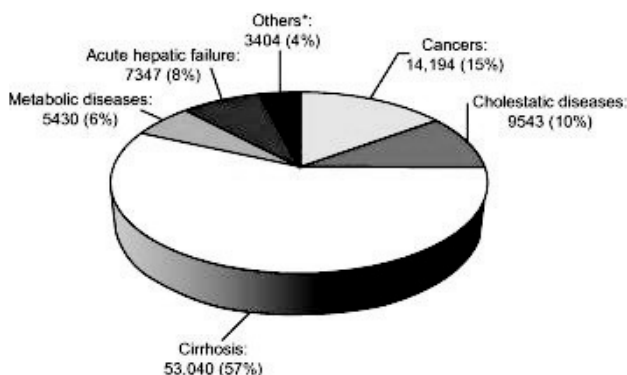
Figure 2: Primary diseases leading to liver transplantation in Europe (01/1988-12/2011). Others: Budd-Chiari, Benign liver tumours or polycystic diseases, parasitic diseases, other. Source EASL Clinical practice Guidelines Liver Transplantation.

The third major indication for liver transplantation is hepatocellular carcinoma (HCC). HCC is the most common primary malignancy of the liver[8]. It occurs on the background of cirrhosis in the majority of patients. Liver transplantation is the treatment of choice in early unresectable HCC. Low rates of recurrence have been reported when Milan criteria are applied for patient selection (solitary HCC with diameter < 5 cm or up to 3 nodules with diameter < 3 cm)[9].

In cirrhotic patients, the severity of liver disease is assessed by the MELD score (based on creatinine, bilirubin and INR) which is a good predictor of short-term pre-transplant mortality risk[10]. If a liver transplantation is considered in a patient, an assessment will also include the presence of comorbidities, a nutritional assessment, a screening for infections and (pre)malignant lesions and a socio-psychiatric assessment with a special focus on the presence of addictions[5]. All transplant candidates should be discussed in multidisciplinary transplant meetings.

1.3.1.2 Organ donation and allocation

In the EU, organs cannot be procured without the consent of donors and/or their relatives. This can be a presumed consent, where every citizen is an organ donor unless "opting-out" actively. In other countries potential donors need to opt-in actively and register as an organ donor.

Donation after brain death (DBD) is the most common type of deceased donation, while donation after circulatory death (DCD) is increasingly used as an additional source of organs for transplantation. DCD is a graft donation from a donor who has suffered an irreversible cardiac arrest and has suffered devastating and irreversible brain injury and may be near death, but does not meet formal brain death criteria. After consent of the relatives, care is withdrawn and donor organs are recovered in the operating room after circulation has seazed. This type of donation does not cause or hasten death. Organs recovered from a donor after cardiac death have some degree of oxygen deprivation during the time after the heart stops beating which can lead to diminished graft survival[11]. Living donation represents only a small part of liver transplantation in the EU[2].

Belgium is a member of Eurotransplant (ET)[12], an organ exchange organization that cooperates with Austria, Croatia, Germany, Hungary, Luxembourg, the Netherlands and Slovenia for organ allocation. Since 2006, allocation is based on the "sickest-first" principle using the MELD scoring. Patients whose severity is not adequately reflected by lab MELD can be requested for an exceptional MELD. The most frequent, eg. HCC, have been identified as standard exceptions[12].

Several strategies have been tried to increase the donor organ pool. A first strategy is the use of extended criteria donors (ECD), also called marginal donors. These donor organs represent

unfavorable characteristics associated with suboptimal post-transplant outcome due to higher risk of poor graft function or due to potential disease transmission[13]. ET defines marginal liver donors as donor age > 65 years, ICU stay with ventilation > 7 days, BMI > 30, steatosis of the liver > 40%, serum sodium > 165 mmol/l, increased transaminases (AST >105 U/L;ALT> 90U/L) and serum bilirubin >3 mg/dl.

Another strategy is the use of DCD liver grafts, as mentioned before, and liver grafts from donors of older age. Transplantation of livers from septuagenarian and octogenarian donors can achieve excellent long-term patient and graft survival for selected HCV negative patients[14]. Steatotic liver grafts show an increased risk of ischemia-reperfusion injury. Although mild macrosteatosis (<30% volume) is not considered to affect the transplant outcome, moderate and severe steatosis can be a contraindication for use of the donor organ[15]. The use of extracorporeal normothermic machine perfusion and machine perfusion-based defatting protocols might change this approach in the near future[16]. Finally the use of HBcAb positive donors grafts is safe in HBsAg positive patients and requires prophylaxis in HBsAg negative patients[13]. The use of HCV positive donor graft is considered safe in HCV positive patients but should be avoided in non-HCV positive recipients as outcome can be affected[17]. However, the implementation of direct acting antiviral drugs might generate arguments to change this approach[18]. The use of organs from donors with previous or current malignancies can occur and is often fatal in immunosuppressed transplant patients. Each case should be carefully considered depending on the exact tumour stage and the disease-free interval[5].

A scoring system based on donor characteristics, the donor risk index (DRI) was developed in 2006 in order to assess the risk of graft failure after liver transplantation[19]. A European variant, the ET-DRI has been developed since[20].

1.3.1.3 Immunosuppression

In contrast to other organs, the liver requires less immunosuppression than other organs. The occurrence of acute cellular rejection (ACR) does seldom lead to graft loss and thus a less strict approach towards immunosuppression is allowed, especially after the first year after liver transplantation[13].

The cornerstone of immunosuppression are calcineurin-inhibitors. Both cyclosporine (CsA) and tacrolimus (Tac) bind to cytoplasmic receptors and the resulting complexes inactivate calcineurin, a pivotal enzyme in T cell receptor signaling[21]. Tac is the drug of choice in 90% of liver transplant patients. Mycophenolic acid is the active metabolite of mycophenolate mofetil (MMF) and is a

selective, non-competitive inhibitor of inosine monophosphate dehydrogenase, and reduces purine synthesis, affecting lymphocyte proliferation. It is used in combination with CNI in order to reduce dosage and side effects of CNI. These side effects will be addresses in the next section. Sirolimus and everolimus are inhibitors of the mammalian target of rapamycin (mTOR) and interact with IL-2 signaling. The side-effects include aftosis, skin lesions, decreased wound-healing and hyperlipidemia[22]. In contrast to CNI mTOR inhibitors do not induce nephrotoxicity.
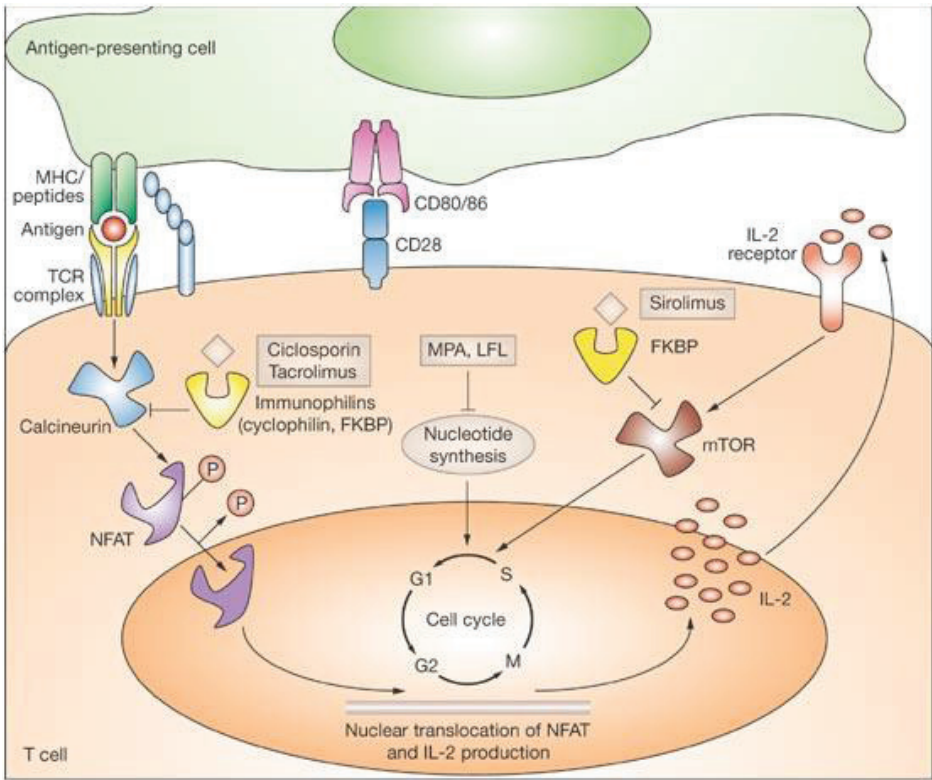
Figure 3: Mechanisms of action of immunosuppressive agents. CNIs (ciclosporin and tacrolimus) bind to their respective immunophilins, and inhibit calcineurin. Calcineurin is then unable to dephosphorylate NFAT, which will prevent translocation of NFAT to the nucleus and thereby production of IL-2. Sirolimus is an mTOR inhibitor. It binds to FKBP and inhibits mTOR, which in turn inhibits transition of the cell cycle from G1 to S phase. MPA and LFL are also cell-cycle inhibitors, and act via inhibition of nucleotide synthesis. *Abbreviations: CNI, calcineurin inhibitor; FKBP, FK506-binding protein; IL-2, interleukin-2; LFL, leflunomide; MHC, major histocompatibility complex; MPA, mycophenolic acid; mTOR, mammalian target of rapamycin; NFAT, nuclear factor of activated T cells; TCR, T cell receptor. Source Samaniego et al.[21]*

Corticosteroids have classically been added to CNI and MMF during the first 3 to 6 months after liver transplantation at the cost of increasing corticoid-related side-effect[23]. Induction agents like IL-2 receptor monoclonal antibodies (daclizumab and basiliximab) are attractive corticoid-sparing alternatives, especially in patients with pre-transplant renal impairment[24].

1.3.1.4 Graft and patient survival

Graft and patient survival after liver transplantation are excellent over the last several years. The chance of one-year survival is close to 90% and the 5-year survival rate is around 70%[2].

Early graft loss can be due to primary non function (PNF). PNF occurs after 2-10%[25–27] of liver transplantations and was first described by Shaw et al.[28] as a situation where "a graft never demonstrated evidence of initial function following transplantation" after exclusion of technical causes and acute cellular rejection needing an urgent retransplantation. PNF requires urgent retransplantation.

In the early post-transplant period surgical complications and infections account for the majority of deaths and graft losses. Surgical complications after transplantation include vascular complications and biliary complications. Vascular problems are mainly arterial and range from acute hepatic artery thrombosis, which can affect graft survival on the short term or more modest vascular impairments that can cause ischemic biliary lesions which can affect the graft survival on the long term[2930] and often lead to retransplantation. In contrast to these non-anastomotic ischemia-type bile duct lesions, anastomotic stenosis can be seen in 4-9% of patients. It is often observed in the first year after liver transplantation. Conventional endoscopic treatment with ERCP with balloon dilatation and use of prosthesis has a success rate of 70-100%[31]. Overall, graft loss occurs in 7-10% of patients and liver retransplantation is the only definite solution for these patients[32].

On the long term, de novo malignancies and cardiovascular diseases are the major reason for death[2]. Recurrence of the underlying liver disease, in particular hepatitis C infection has had a dramatic effect on transplant outcome. The advent of newer DAA's has the potential to eliminate this problem[33]. ACR occurs in 15-30% of liver transplant recipients[34] but can be successfully treated in the majority of patients. In contrast, chronic ductopenic rejection is difficult to treat and can lead to graft loss[35].

This has lead to a paradigm shift in liver transplantation. The discovery of cyclosporine in 1971 enabled the development of liver transplantation from an experimental procedure to the standard of care for end stage liver disease as ACR could be treated effectively. Today, over-immunosuppression

is inducing (opportunistic) infections in the early post-transplant period and the side-effects of CNI cause cardiovascular disease and cancer development in the late posttransplant period, both leading to impaired patient survival.

One of the keys to bring liver transplantation to a higher level and to improve patient survival in the next years will be the development of strategies to identify patients who are tolerant and who can survive without any immunosuppression. Indeed, up to 20% of patients show spontaneous tolerance[36]. Another strategy will be the development of biomarkers that can guide clinicians in the use of immunosuppression, in order to use the lowest possible dosage. Finally, strategies for induction of tolerance could radically change our medical practice[37].

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1.3.2 BIOMARKERS FOR ACUTE CELLULAR REJECTION

The current gold standard for the diagnosis of acute cellular rejection (ACR) is a liver biopsy and this has remained unchanged until now, despite many attempts to develop non-invasive biomarkers that could diagnose ACR with a high accuracy. Unfortunately, after performing a literature review we had to conclude that the perfect biomarker has not been found (yet).

Biomarkers for the diagnosis of acute cellular rejection in liver transplant recipients: A review

Xavier Verhelst, Roberto Troisi, Isabelle Colle, Anja Geerts, Hans Van Vlierberghe

1.3.2.1 ABSTRACT

The gold standard for the diagnosis of acute cellular rejection (ACR) is a liver biopsy. The quest for an alternative non-invasive biomarkers has been long and is ongoing. However, an efficient and useful biomarker has not been developed yet. In this manuscript, we review all possible candidate biomarkers that have been studied in recent years, starting with cytokines and ending with an overview of different newly discovered "omics". Promising paths are being explored but a valid non-invasive biomarker has not been discovered yet.

1.3.2.2 INTRODUCTION

Since the first liver transplantation in 1963 by Starzl[1], liver transplantation has been considered a standard of care treatment for end-stage liver diseases with excellent long-term survival and accepted morbidity and mortality rates. However, the early post-transplant period can be troubled by a variety of complications, including delayed graft function, hepatic artery and vein thrombosis and biliary complications. The most common complication in this period is acute cellular rejection (ACR), occurring in 20–40% of patients[2]. Diagnosis of ACR is based upon clinical suspicion on one hand, raised by nonspecific symptoms like malaise, fever, abdominal pain, hepatomegaly and increasing ascites, and by laboratory abnormalities on the other hand, including elevation of serum aminotransferases, alkaline phosphatases, g-glutamyl transferases and bilirubin levels. However, these signs and symptoms are non-specific and do not correlate with the severity of rejection[3]. Confirmation requires a liver biopsy, considered the gold standard[4], which is costly and causes morbidity and mortality[5]. Despite correct counseling, a liver biopsy can cause anxiety in many patients. Furthermore, even if a liver biopsy is the gold standard, diagnostic accuracy is challenged by sampling error and interpretation is not always straightforward[6]. In this manuscript, we review the possible non-invasive diagnostic tools for the diagnosis of ACR. Animal studies will not be discussed in this review.

1.3.3.3 ACUTE CELLULAR REJECTION

ACR is a T-cell-dependent immune response directed against donor tissues resulting from the recognition of alloantigens by recipient T cells[7] followed by T-cell activation and proliferation. The *primum movens* of the ACR is the binding of foreign antigens from newly transplanted organs to

antigen-presenting cells of the recipient resulting in an activation of recipient T cells. The activated T cells in turn release interleukin 2 (IL-2) which binds to the IL-2 receptors (IL-2R) only expressed on the surface of activated T cells. The IL-2R is composed of three transmembrane protein subunits, a (CD 25), b (CD 122) and g (CD 132). The first is specific to IL-2R. Binding to a and b subunits is a crucial step in T-cell activation and propagation[7,8]. This explains why IL-2 can be considered a catalyzer of the cellular immune response and is an attractive therapeutic target. For instance, basiliximab (a chimeric monoclonal antibody targeting CD25) and daclizumab (a humanized monoclonal antibody with the same target) are used as prophylactic agents in the early post-transplant period[9,10]. In this cascade of events, an interaction between the CD28 molecule and the B7 ligand is necessary as a second signal for optimal T-cell activation and IL-2 production[11]. This ultimately leads to infiltration of the graft by host T cells and damage of the graft, as can be appreciated by histological assessment of a liver biopsy. This is typically characterized by portal inflammation, bile duct inflammation damage and venous endothelial inflammation.

1.3.3.4 BIOMARKERS FOR ACR

The first question should address what a good biomarker is. The Biomarkers Definitions Working Group defined a biomarker as "a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention"[12]. A perfect diagnostic biomarker for ACR should be highly sensitive and specific, non-invasive, rapidly available and budget-friendly. The second question should answer if a potential biomarker has proven clinical utility and has been externally validated. Indeed, many potential biomarkers have been reported to have diagnostic potential, but few have been validated. Validation criteria for ACR are not available, but we were inspired by the minimal requirements for the validation of noninvasive fibrosis markers according to the French National Authority for Health (Haute Autorité de Santé) as adapted by Ratziu[13]. Based on this, we propose a set of five criteria assessing the intrinsic quality of the biomarker for ACR and the quality of the study report. These criteria are: (i) sensitivity, specificity, area under the receiver–operator curve (AUROC); (ii) discrimination from other events, including cytomegalovirus (CMV) infection and recurrence of hepatitis C virus (HCV) infection in the liver graft; (iii) easily available high throughput test; (iv) sufficiently large sample size with prospectively analyzed patients; and (v) one independent validation.

Both biomarkers that are diagnostic for ACR and biomarkers that are prognostic for ACR and identify patients at increased risk for ACR development can be identified.

PAST AND PRESENT BIOMARKERS FOR ACR

Serum markers related to inflammation

Rising of liver enzymes after transplantation is often the first reason to suspect ACR. However, sensitivity and specificity of liver enzymes are low and these enzymes cannot differentiate ACR from others complications. The AUROC for aspartate aminotransferase, alanine aminotransferase (ALT), g-glutamyltransferase, total bilirubin and conjugated bilirubin is approximately 0.5. For alkaline phosphatase, the AUROC is slightly better (0.69) and although this value reached statistical significance, the clinical significance remains doubtful[3]. The first potential biomarkers studied were cytokines and other proteins related to the inflammatory response. Growing insight into the immunological basis of ACR accompanied the study of these cytokines as biomarkers for ACR. For example, a rise of CD28 expression up to 6 days before diagnosis of ACR has been observed[14,15]. A French group studied the expression of CD25, CD28 and CD38 on CD3+, CD4+ and CD8+ cells, respectively. The mean frequencies of CD28 and CD38-expressing T cells (in peripheral blood mononuclear cells) were significantly higher in patients with acute rejection, whereas the frequency of CD25-expressing T cells did not differ significantly between ACR and non-ACR patients[16]. This expression decreased after anti-rejection therapy. Although in this study the CD28 and CD38 expression levels did not change in patients suffering from an acute CMV infection, other data report an expression of this same CD28 and CD38 expression during CMV infection[17], limiting its clinical use. Soluble IL-2R (sIL-2R) levels in serum are increased as early as 10 days before the diagnosis of ACR but also increase in cases of CMV infection[18,19], bacterial infections and cholangitis[20,21]. However, if the ratio of the post-transplant level divided by the pre-transplant level of SIL-2R was measured in combination with the levels of CD8, a more pronounced elevation of both levels was observed during CMV infection in comparison with ACR, where levels of CD8 are not increased[22]. Soluble tumor necrosis factor (TNF) receptor II (sTNF-RII), released upon stimulation of T-helper (Th)1 lymphocytes, and IL-10, a counter regulatory Th2 cytokine, increase as well during ACR as during serious infections. Neopterin, an intermediate of tetrahydrobiopterin synthesis produced by interferon (IFN)-g-activated macrophages, increased at the onset of ACR only in steroid resistant patients. The pro-inflammatory cytokines IFN-g, IL-1b, IL-4 and IL-6 were not of any use[20]. IL-6 is an inducer of the hepatic synthesis of a myriad of acute phase proteins. Kita *et al*. observed in contrast a marked rise of IL-6 during ACR and during infection, however, the rise pattern was distinguishable between both[23]. Interleukin-15 is produced by non-lymphatic cells including macrophages, dendritic cells and epithelial cells. Its biologic activities are similar to those of IL-2. Plasma levels of IL-15 are increased during ACR, particularly during steroid-resistant ACR and during chronic rejection[24]. Also, TNF-a, currently used on a daily basis in clinical settings as a marker of infection, once was proposed as a potential

biomarker for ACR. Levels of TNF-a are elevated during ACR but cannot discriminate ACR from infection[25]. Beta2-Microglobulin is a low molecular weight protein included in the major histocompatibility complex class I complex required for its expression. ACR in cardiac and renal transplant patients is associated with increased levels of b2-microglobulin. The same was observed in liver transplantation, but this marker could not differentiate ACR from infectious complications[26-28]. The infiltration of leukocytes into the allograft during ACR is regulated by the expression of adhesion molecules[29]. An increase of intercellular adhesion molecule 1 (ICAM-1) and E-selectin in serum was observed in relation to ACR. However, neither E-selectin[30,31] nor ICAM-132 could differentiate ACR from an infectious episode. A differentiation was seen between patients with ACR and CMV infection, where ICAM-1 levels did not increase[33]. Serum vascular cellular adhesion molecule 1 (VCAM-1) and E-selectin were associated to the extent of preservation and reperfusion injury in one study[33], but were also attributed to ACR in another group[31]. These data could not be reproduced by other research groups[34]. We also have to take into account that these values do increase during the first days after transplantation, probably due to a rebound phenomenon that reflects immunological activation due to surgery and organ conservation[31,34]. In pediatric patients, a rise in plasminogen activator inhibitor 1 was noticed before ACR and was suggested as a candidate biomarker. Validation in a larger cohort has not been reported.

A Japanese group developed an enzyme-linked immunoassay (ELISA) for the measurement of serum human myeloid-related protein complex (MRP8/14). MRP8/14 is expressed in activated human granulocytes and monocytes in the inflammatory phase and is involved in the inflammation-related calcium-dependent activation. In liver transplant recipients, a clear association was observed between serum levels and ACR, however, sensitivity and specificity were not published. Furthermore, there is no information regarding the expression of MRP8/14 during infectious complications[36]. However, in kidney transplant recipients, MRP8/14 was also increased during non-viral infections, but in combination with procalcitonin a discrimination was possible[37]. It seems obvious that the role of the adaptive immune response is well established in the occurrence of ACR. On the other hand, the role of the innate immunity is less clear. The role of Toll-like receptors (TLR), mediators of innate immunity, was studied in ACR. Patients experiencing ACR had significantly higher levels of TLR4 and a greater capacity to produce the pro-inflammatory cytokines TNF-a and IL-6 before transplantation, but had a downregulation of the TLR4 pathway if they experienced rejection. In contrast, there was no correlation between TLR2 levels and rejection[38]. Apoptosis is an important mechanism of cell death during ACR and this is mediated via Fas ligand. Increased serum levels of soluble Fas antigen have been detected in patients during ACR[39]. Finally, several studies illustrate that blood eosinophilia

could be an interesting biomarker for ACR[40,41]. In one study, a positive predictive value of 82% was found but, more interestingly, a negative predictive value of 86%[42]. However, the response was less clear in patients who received steroids and in HCV-infected patients. Although most of these markers do prove a relationship with ACR, only five could be retained as valuable because these showed a clear relationship with the appearance of ACR, could differentiate from other post-transplant complications and were performed on prospective patient series. The characteristics are summarized in Table 1.

Serum markers not related to inflammation (Table 2)

Other potential biomarkers were a-glutathione S-transferase (a-GST) and p-glutathione S-transferase (p-GST). GST are a family of multifunctional detoxifying enzymes that are implicated in the conjugation of glutathione with several compounds. a-GST is a low molecular weight protein widely present in the hepatocyte cytosol with a short half-life. Plasma values increase rapidly in case of ACR but lack sensitivity and specificity to claim clinical usefulness[46-49]. p-GST is an isoenzyme exclusively found in the biliary epithelium of the liver that was also tested but was not found to be related to ACR[49]. A single report mentions the elevation of carbohydrate antigen (CA)-19.9, routinely used as a tumor marker for pancreatic and bile duct malignancies[50]. The rise of CA-19.9 might be explained by the release of CA-19.9 due to cell damage caused by the inflammatory reaction. In a rat model, ceruloplasmin was shown to be reduced during ACR. The underlying physiopathology is unclear[55]. A group from Kings College London observed an increase of acid-labile nitrosocompounds (NOx) during ACR which correlated with rejection severity and with response to treatment; in contrast, there was no correlation with nitrate (NO3 –) levels[51]. However, another group found a clear relationship between ACR and nitrate levels[52]. The clinical usefulness remains unclear. In a small patient series, serum amyloid A protein (SAA) was significantly increased during the appearance of ACR[53], but SAA could not differentiate ACR from infections[21]. N-protein-bound conjugated bilirubin has been reported as a helpful biomarker for the diagnosis of ACR, however, sensitivity and specificity were not satisfactory[26,54]. These data are summarized in Table 2.

Bile markers

Conflicting results (summarized in Table 3) have been published regarding the validity of bile markers. Umeshita *et al*. found a clear rise of bile IL-6 after ACR and a decrease in the case of a successful treatment[56], but this was also seen in cholangitis. Biliary IL-8 also increases in the early stage of ACR but specificity is not higher than serum cytokine markers[44]. Adams *et al*. reported elevated IL-2R in the bile of patients with ACR, with higher sensitivity and specificity than in serum[19]. Elevated levels of ICAM-1 in bile have been observed in ACR[32,45] but in another study the same was

observed during the appearance of infectious complications[44]. On the other hand, another group could not find a relationship between ICAM-1 and VCAM-1 in bile and ACR. A Japanese group discovered (in a small group of five patients with biopsy-proven ACR) a raise of alanine aminopeptidase N 3 days before the appearance of ACR[57]. A major drawback in clinical practice is the bile collection that requires the position of a T tube.

Ascites markers

In a small series of pediatric liver transplant patients, an increase of the IL-1R antagonist was observed in ascites 48 h before rejection (see Table 4) [58]. Ascites collection after transplantation requires puncture or the position of a peritoneal drainage which can cause infections. Furthermore, ascites is not always present after transplantation.

FUTURE BIOMARKERS

The perpetual advance of the last decade has opened new perspectives for the discovery of biomarkers, particularly in the field of genomics, transcriptomics, proteomics and metabolomics. Today, two non-invasive tests are US Food and Drug Administration approved and commercially available in the field of organ transplantation. The first, AlloMap (XDx, South San Francisco, CA, USA), predicts the absence of ACR after heart transplantation[59]. The second, the immune function test ImmuKnow (Cyclex Incorporated, Columbia, MD, USA), provides a preliminary evaluation of the degree of activation of CD4 T cells by measuring the ability of CD4+ T cells to respond to *in vitro* mitogenic stimulation by quantitating adenosine triphosphate production. This result can help to titrate the immunosuppressive therapy after kidney transplantation[60]. This announces a paradigm shift from measuring serum drug levels, only providing an estimation of the immunosuppressive state to the direct measurement of the *in vivo* actual immune function. For transplant hepatologists, ImmuKnow has not been approved yet, but data are exciting. Several trials have demonstrated that this assay could identify patients with a low immune response (at risk of infections) and patients with a high immune response (at risk of ACR)[61-63]. Immuknow also can distinguish between ACR and recurrent HCV infection[64], where patients experiencing HCV recurrence showed a significantly lower immune response than patients developing ACR.

Genomics

Genome-wide association studies have identified loci associated with an increased susceptibility to ACR, for example, the copy number variation in the *CCL3L1* gene[65] or to poor allograft survival[66], but biomarkers associated with the acute event of ACR are not available at this moment, in contrast to

Table 1 Overview of valuable serum biomarkers related to inflammation in acute cellular rejection after transplantation

Biomarker	Reference	Performance (sensitivity/ specificity/AUROC)	Discrimination from other events	Assay	Prospective population, sample size (n)	Independent validation
CD28	Boleslawski 2008[16]	Positive performance (NR/NR/NR)	Yes	Flow cytometry	Yes, 52	Yes
	Minguela 2006[15]	Positive performance (NR/NR/NR)	Yes	Flow cytometry	Yes, 182	Yes
	Blanco-Garcia 2011[43]	Positive performance (88%/61%/0.72)	Yes	Flow cytometry	Yes, 150	Yes
CD38	Boleslawski 2008[16]	Positive performance	Yes	Flow cytometry	Yes, 52	No
IL-4	Platz 1996[20]	Positive performance (50%/NR/NR)	No	Enzyme immune assay	Yes, 81	Conflicting results
	Kita 1994[23]	Positive performance (76%/NR/NR)	Yes	ELISA	Yes, 24	Conflicting results
ICAM-1	Warle 2003[44]	Positive performance (NR/NR/NR)	Yes	ELISA	Yes, 45	Conflicting results
	Mueller 1997[30]	Positive performance (NR/NR/NR)	No	Enzyme immune assay	Yes, 85	Conflicting results
	Navarro 1998[34]	Negative performance	No	ELISA	Yes, 11	Conflicting results
	Goto, 1998[31]	Positive performance (NR/NR/NR)	No	ELISA	Yes, 29	Conflicting results
	Lang 1995[32]	Positive performance (NR/NR/NR)	No	ELISA	Yes, 11	Conflicting results
	Ninova 1995[33]	Positive performance (NR/NR/NR)	Yes	Enzyme immune assay	Yes, 13	Conflicting results
	Adams 1993[45]	Positive performance (NR/NR/NR)	No	ELISA	Yes, 61	Conflicting results
Eosinophilia	Barnes 2003[42]	Positive performance (45%/80%/NR)	Yes	Cell count	Yes, 101	Yes
	Foster 1989[40]	Positive performance (92%/82%/NR)	Not reported	Cell count	Yes, 60	Yes

All cases of acute cellular rejection were confirmed by liver biopsy.
AUROC, area under the receiver–operator curve; ELISA, enzyme-linked immunoassay; ICAM-1, intracellular adhesion molecule-1; IL, interleukin; NR, not reported.

Table 2 Overview of serum markers not related to inflammation in acute cellular rejection after transplantation

Biomarker	Reference	Performance (sensitivity/specificity/AUROC)	Discrimination from other events	Assay	Prospective population, sample size (n)	Independent validation
α-GST	Dickson 1999[46]	Positive performance (79%/20%/NR)	NR	Enzyme immunoassay	Yes, 44	Conflicting results
	Trull 1994[47]	Positive performance (100%/28%/NR)	No	Enzyme immunoassay	Yes, 45	Conflicting results
	Nagral 1998[48]	Positive performance (38.8%/NR/NR)	NR	Enzyme immunoassay	Yes, 23	Conflicting results
	Platz 1997[49]	Positive performance (NR/NR/NR)	Yes	Enzyme immunoassay	Yes, 81	Conflicting results
π-GST	Dickson 1999[46]	Negative performance	NR	ELISA	Yes, 44	Conflicting results
	Platz 1997[49]	Positive performance	NR	Enzyme immunoassay	Yes, 81	Conflicting results
CA-19.9	Sameshima 1992[50]	Positive performance (NR/NR/NR)	No	ELISA	Yes, 26	No
NOx	Devlin 1994[51]	Positive performance (NR/NR/NR)	Yes	Chemiluminescence	Yes, 50	No
Nitrate	Devlin 1994[51]	Negative performance (NR/NR/NR)	No	Chemiluminescence	Yes, 50	Conflicting results
	Fabrega 1997[52]	Positive performance (NR/NR/NR)	NR	Not reported	Yes, 20	Conflicting results
Serum amyloid A	Feussner 1994[53]	Positive performance (67%/96%/NR)	No	LIA	Yes, 12	Yes
	Lalli 1992[21]	Positive performance (NR/NR/NR)	No	ELISA	Yes, 20	Yes
Non-protein bound conjugate bilirubin	Cox 1987[54]	Positive performance (78%/77%/NR)	NR	HPLC	Yes, 80	No
Neopterin	Platz 1996[20]	Positive performance (NR/NR/NR)	No	Enzyme immunoassay	Yes, 81	No

All cases of acute cellular rejection were confirmed by liver biopsy.
α-GST, glutathione S-transferase; AUROC, area under the receiver–operator curve; CA, carbohydrate antigen; HPLC, high-performance liquid chromatography; LIA, laser immunonephelometric assay; NOx, nitrosocompounds; NR, not reported; π-GST, π-glutathione S-transferase.

Table 3 Overview of bile markers related to inflammation in acute cellular rejection after transplantation

Biomarker	Reference	Performance (sensitivity/ specificity/AUROC)	Discrimination from other events	Assay	Prospective population, sample size (n)	Independent validation
IL-6	Umeshita 1996[56]	Positive performance (NR/NR/NR)	Yes	Enzyme immune assay	Yes, 51	No
IL-8	Warle 2003[44]	Positive performance (NR/NR/NR)	Yes	ELISA	Yes, 45	No
IL-2 receptor	Adams 1989[19]	Positive performance (NR/NR/NR)	Not reported			
ICAM-1	Navarro 1998[34]	Negative performance (NR/NR/NR)	Not reported	ELISA	Yes, 11	Conflicting results
	Lang 1995[32]	Positive performance (NR/NR/NR)	Yes	ELISA	Yes, 11	Conflicting results
	Adams 1993[45]	Positive performance (NR/NR/NR)	Yes	ELISA	Yes, 43	Conflicting results
VCAM-1	Navarro 1998[34]	Negative performance (NR/NR/NR)	Not reported	ELISA	Yes, 11	No
Alanine aminopeptidase	Kim 2011[57]	Positive performance (NR/NR/NR)	Not reported	Enzyme immune assay	Yes, 9	No
N						

All cases of acute cellular rejection were confirmed by liver biopsy.
AUROC, area under the receiver–operator curve; ELISA, enzyme-linked immunoassay; ICAM-1, intracellular adhesion molecule-1; IL, interleukin; NR, not reported; VCAM-1, vascular cell adhesion molecule-1.

Table 4 Overview of ascites markers related to inflammation in acute cellular rejection after transplantation

Biomarker	Reference	Performance (sensitivity/specificity/AUROC)	Discrimination from other events	Assay	Prospective population, sample size (*n*)	Independent validation
IL-1 receptor antagonist	Ganschow 2000[58]	Positive performance (NR/NR/NR)	Yes	Enzyme immune assay	Yes, 33	No
IL-2 receptor	Ganschow 2000[58]	Negative performance	Yes	Enzyme immune assay	Yes, 33	No
IL-6	Ganschow 2000[58]	Negative performance	Yes	Enzyme immune assay	Yes, 33	No

All cases of acute cellular rejection were confirmed by liver biopsy.
AUROC, area under the receiver–operator curve; IL, interleukin; NR, not reported.

the field of kidney transplantation where different promising agents have been identified (see Table 5) [67].

Proteomics

A plethora of proteins are involved in the immune response of ACR. Proteomic analysis of serum seems a very attractive and promising path to the identification of valid biomarkers. Proteomic research in this field has contributed to the better understanding of these processes. Numerous patents have been taken for diagnostic proteomic-based tools, recently reviewed by Fiorini *et al.* [73], indicating the momentum present in this research area. Massoud *et al.*[68] identified 41 serum proteins differentially abundant in the serum of ACR patients. Seven of them (serum amyloid A, complement component 4 [C4], fibrinogen, complement component 1q [C1q], complement component 3, heat shock protein [HSP]-60 and HSP-70) were turned into an ELISA-based assay. C4 and C1q were both independent predictors of ACR. The best diagnostic performance was achieved by C4. Using a cut-off level determined by the researchers, sensitivity was 97%, specificity 62% with a positive predictive value of 74% and a negative predictive value of 94%. Combining C4 levels with ALT levels higher than 70 IU/mL improved these results to 96%, 81%, 86% and 94%, respectively. However, the study cohort included only 16 patients and should be confirmed in larger multicenter trials.

In rats undergoing liver transplantation, eight proteins involved in metabolism were downregulated in rats with ACR, including sulfite oxidase, adenosine triphosphate (ATP) synthase subunit-a, aldehyde dehydrogenase, ATP synthase subunit-b, NADH dehydrogenase-1a, putative L-aspartate dehydrogenase, ketohexokinase and 3-hydroxyisobutyrate dehydrogenase[74].

Transcriptomics

Kobayashi *et al.* observed that the mRNA level of IFN regulatory factor 1 and guanylate-binding protein 2 (GBP2) in leukocytes, both related to T-cell-mediated immune response, were upregulated during ACR but only GBP2 showed statistical significance[71]. The same research group also discovered different transcriptome patterns for ACR in patients with concomitant hepatitis C recurrence, compared to patients with isolated hepatitis C recurrence. Liver injury is associated with release of hepatocyte-derived microRNA (miR). ACR is associated with a rise of miR-122 and miR-148a in serum.

Their elevation is high during ACR and starts before the rise of transaminases[70]. Reverse transcription polymerase chain reaction in cells obtained from the organ perfusate revealed lower levels of HSP-70 mRNA expression in patients experiencing ACR, in comparison to patients without ACR, suggesting a protective role of HSP-70 expression. There was a correlation between the amount of HSP-70 mRNA

expression in these cells and liver biopsies[69]. This may represent a prognostic factor, but has no diagnostic potential at this moment.

Metabolomics

Metabolomics is the quantitative measurement of dynamic multiparametric metabolic responses of living systems to pathophysiological stimuli or genetic modification[75]. Wu *et al*. found distinct metabolomic profiles in rats with ACR after allogenic transplantation correlating with histological changes[76]. In a case report, very distinct metabolomic profiles obtained by proton nuclear magnetic resonance spectroscopy were observed during primary dysfunction of the liver, as early as 2 h after transplantation[72].

1.3.3.5 DISCUSSION

We reviewed all potential biomarkers that have been evaluated as a diagnostic marker for ACR. In the first category of "older" biomarkers, we identified 31 molecules in serum, six in bile and three in ascites. Neither bile- nor ascites-based biomarkers performed better than serum-based biomarkers and should not be taken into account considering the practical concerns for sample collection. The first group of older serum biomarkers was related to inflammation, and contained mainly inflammatory cytokines. Although many of these cytokines show a rise during ACR, they are not useful as biomarkers because they cannot differentiate ACR from other complications, especially infections, that occur during the early post-transplant period and require tailored treatments. We could retain only five valuable biomarkers in this group (CD28, CD38, IL-4, ICAM-1 and eosinophilia), summarized in Table 1. However, even these markers demonstrate important shortcomings, for example, results for ICAM-1 were conflicting. The main shortcoming is the lack of validation in large multicenter studies. In the newer biomarkers of the different "omic" families, the situation is more promising. Only a few reports are available, but they show promising high sensitivity and specificity values, the best being the combination of a proteomic-based ELISA focusing on the appearance of C4 in combination with ALT[68]. This combination claims a sensitivity of 96% and a specificity of 81% with an AUROC of 0.88. However, this trial was based on only 16 patient samples and was not externally validated. Another important clinical concern is the differentiation of ACR from other post-transplantation events like CMV infection, sepsis and HCV recurrence. In the majority of tests, these data are not reported or yield conflicting results. A particular clinical problem is HCV recurrence. The development of fibrosis and cirrhosis in transplanted patients occurs at an accelerated rate compared to immunocompetent patients. As a result, cirrhosis occurs in approximately 25% of those

Table 5 Overview of new biomarkers in acute cellular rejection after transplantation

Biomarker	Reference	Performance (sensitivity/specificity/AUROC)	Discrimination from other events	Assay	Prospective population, sample size (n)	Independent validation
Immuknow	Kowalski 2006[61]	Positive performance odds ratio: 30	Yes		Yes, 504	Yes
	Hashimoto 2010[64]	Positive performance (86%/91%/NR)	Yes		Yes, 54	Yes
	Dong 2011[62]	Positive performance (86%/81%/NR)	Yes		Yes, 83	Yes
Genomics						
CCL3L1	Li 2011[65]	Positive performance (73%/45%/0.62)	Not reported	RT–PCR	Yes, 266	No
Proteomics						
C4*	Massoud 2011[68]	Positive performance (97%/62%/NR)	Not reported	ELISA	Yes, 16	No
C4* + ALT	Massoud 2011[68]	Positive performance (96%/81%/0.88)	Not reported	ELISA	Yes, 16	No
Transcriptomics						
HSP-70 mRNA (organ perfusate)	Flohe 1998[69]	Positive performance (NR/NR/NR)	Yes	RT–PCR	Yes, 14	No
HDmiR	Farid 2012[70]	Positive performance (NR/NR/NR)	Not reported	RT–PCR	Yes, 45	No
IRF1 mRNA	Kobayashi 2010[71]	Positive performance (NR/NR/NR)	Yes	RT–PCR	Yes, 86	No
GBP-2/GAPDH	Kobayashi 2010[71]	Positive performance (63%/85%/NR)	Yes	RT–PCR	Yes, 86	No
Metabolomics						
Distinct profiles**	Serkova 2007[72]	Positive performance (NR/NR/NR)	Not reported	h-NMR spectroscopy	1	No

All cases of acute cellular rejection were confirmed by liver biopsy.

*ELISA comprising SAA, C4, fibrinogen, C1q, C3, HSP-60 and HSP-70. **Increase of glutamine, methionine, citrate.

AUROC, area under the receiver–operator curve; ELISA, enzyme-linked immunoassay; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GBP-2, guanylate-binding protein 2; h-NMR, proton nuclear magnetic resonance spectroscopy; HDmiR, hepatocyte-derived microRNA; HSP, heat shock protein; IRF1, interferon regulatory factor-1; NR, not reported; SAA, serum amyloid A protein; RT–PCR, reverse transcriptase polymerase chain reaction.

transplanted for HCV within a median of 5 years[77]. It can be challenging to differentiate ACR from HCV recurrence. However, only six out of all the markers reviewed in this article were tested for their ability to differentiate patients with ACR from patients with HCV recurrence[15,25,27,42,64,71]. Only Immuknow was able to differentiate between both[64]. CD28 yielded conflicting results[15,43]. Measurement of guanylate-binding protein 2/glyceraldehydes 3-phosphate dehydrogenase showed a trend toward differentiation, but this was not statistically significant[71]. Microarray studies are a promising path to distinguish between ACR and HCV recurrence. Differential gene expression has been observed in liver tissue between patients with ACR, HCV recurrence[78] and the absence of both[79]. These different gene expression patterns reflect distinct pathophysiological pathways.

Genomic analysis will be helpful for the further elucidation of these pathways. However, at this moment, microarray-based tests performed on serum are not available. A rare clinical entity is antibody-mediated rejection (AMR). It is caused by preformed donor-specific human leukocyte antigen antibodies (DSA) and complement activation, and is defined by graft dysfunction, histological evidence of acute tissue injury, complement component 4 deposition in the vascular walls and elevated DSA mean fluorescence intensity (MFI) [80]. AMR is often treatment resistant due to the combination of both cellular and humoral mechanisms and often results in graft loss in kidney and heart transplant recipients[81,82]. The clinical significance of AMR after liver transplantation is still a matter of debate. Recent work observed DSA in 22.2% of a large prospective liver transplant cohort, without affecting rejection rates[83]. However, several case reports in ABO blood group-compatible liver transplants have been reported with poor graft outcome[84-86]. These data suggest that in steroid-resistant patients with acute rejection, measurement of DSA titers can lead to the identification of AMR and trigger appropriate treatment[87]. If a promising biomarker has been identified, it should be rapidly validated in large multicenter trials that are able to recruit a sufficient amount of patients and events to achieve enough power. Discrimination from other events should be tested as well. This is probably where former research groups have failed.

1.3.3.6 CONCLUSION

THE SEARCH FOR novel biomarkers for the diagnosis of ACR after liver transplantation has progressed in parallel with the discovery of new insights in the pathogenesis of rejection. From cytokines over genomics to metabolomics, the perfect biomarkers able to challenge the liver biopsy have not been discovered yet. However, new techniques and the discovery of new insights in all kind of "omics" have the potential of bearing this long-awaited non-invasive biomarker.

1.3.3.7 REFERENCES

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1.4 PROTEIN N-GLYCOSYLATION AND GLYCOMICS

1.4.1 GENERAL INTRODUCTION

1.4.1.1 Protein N-Glycosylation

In eukaryotic cells, proteins are frequently modified with complex glycan structures. This process, called glycosylation, is the most frequent posttranslational modification[1]. Three major types of glycosylation have been observed : *N*-linked glycosylation of asparaginases[1], *O*-linked glycosylation of serine and threonine[2] and glycosylphosphatidyl inositol derivatization of the carboxyl-terminal carboxyl groups[3]. Here, we will focus on the *N*-glycosylation of humans.

N-glycosylation is essential for cell viability. It is strictly regulated by a multitude of enzymes[4] and at least half of the genes functioning in this biosynthetic pathway have been conserved through evolution. *N*-glycosylation is the principal posttranslational modification of serum proteins. These glycan structures are involved in diverse biological processes including protein folding and conformation, cell structure and stability and cell-matrix and cell-cell interaction[5].

Alterations in the abundance of particular N-glycans reflect an altered physiological state. This makes N-glycans particularly attractive for biomarker development. Furthermore, N-glycoproteins are highly regulated during growth and differentiation and alterations in protein N-glycosylation patterns correlate with developmental programs like morphogenesis, proliferation and apoptosis[6].

1.4.1.2 Biosynthesis of N-Glycans

Protein N-glycosylation occurs along the secretory pathway and is localized in the endoplasmic reticulum (ER) and the Golgi apparatus[7]. N-glycan synthesis can be divided in 3 major steps (Figure 1).

- Formation of a lipid-linked precursor oligosaccharide
- en bloc transfer of the oligosaccharide to the polypeptide
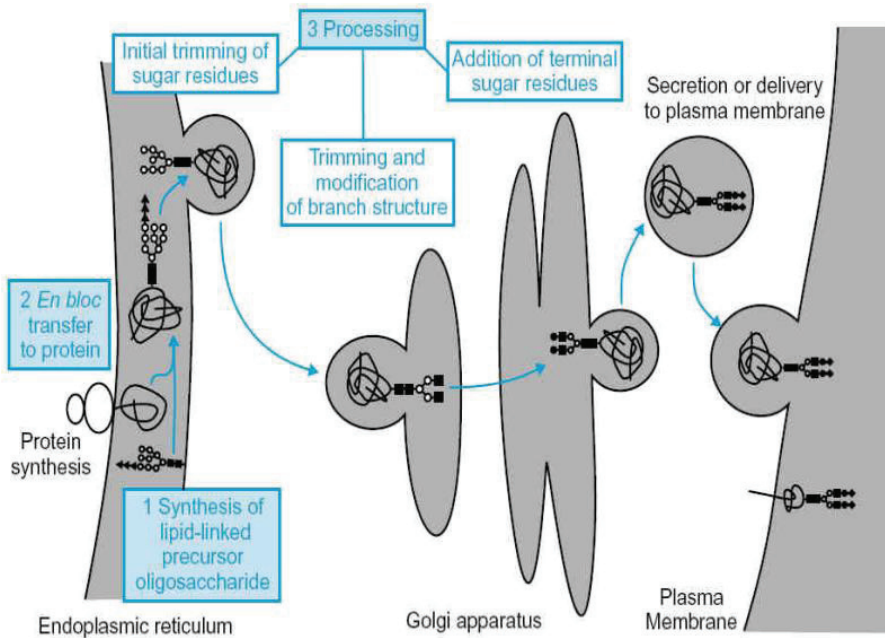- processing of the oligosaccharide[8]

Figure 1. Overview of the pathway for glycoprotein biosynthesis and its location within a cell. (Source : Taylor and Drickamer. Introduction to Glycobiology. Oxford 2011).

Formation of a lipid-linked precursor oligosaccharide

The donor that initiates N-linked glycan synthesis is a $Glc_3Man_9GlcNAc_2$ structure attached to the lipid dolichol through a pyrophosphate linkage[9]. Assembly of a glycan on the dolichol head group takes place in two phases. The first phase occurs on the cytoplasmic side of the ER membrane, whereas the second phase occurs in the lumen. UDP-GlcNAc and guanosine diphosphate mannose (GDP-Man) are used as sugar donors for the attachment of the two GlcNAc residues and the first 5 mannose residues. Then, the lipid-linked glycan is translocated across the membrane and becomes inaccessible to cytoplasmic enzymes (Figure 2)[7,9].

On the luminal side of the ER membrane, further sugars are added using dolichol-linked sugars as donors. These are synthesized on the cytoplamic face of the ER membrane and translocated across the membrane.
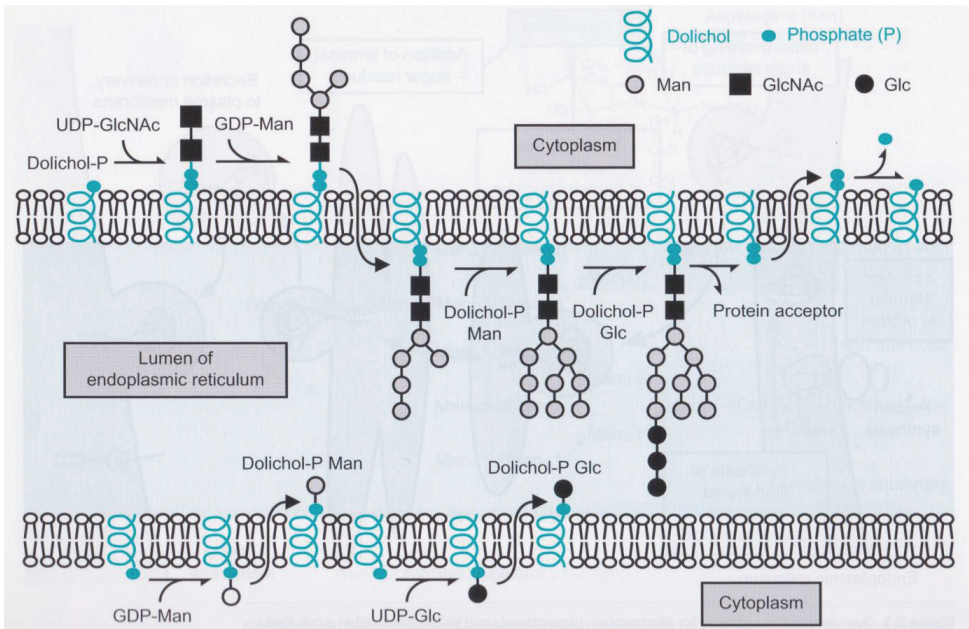
Figure 2. Pathway for generation of the dolichol-linked oligosaccharide donor for protein N-Glycosylation. (Adapted from Taylor and Drickamer. Introduction to Glycobiology. Oxford 2011).

En bloc transfer of the oligosaccharide to the polypeptide

The dolichol-linked precursor oligosaccharide is transferred to a asparagine residue in the sequence Asn-X-Ser or Asn-X-Thr, where X can be any amino acid except proline. N-linked glycans are found at the surface of proteins. Considering that N-linked glycosylation only is initiated in the lumen of the ER, N-glycans are only found in secreted proteins and in the portions of transmembrane proteins that face the lumen[1].

The completed dolichol-bound precursor glycan is transferred to a polypeptide acceptor by oligosccharyltransferase (Figure 1). Exoglycosidases remove monosaccharides from the non-reducing end on the glycan[7]. This proces is located in the endoplasmic reticulum.

Lysosomal proteins are also synthesized in the ER and specifically transported through the Golgi apparatus to the trans-Golgi network, from which transport vesicles bud to deliver them to the endosomal/lysosomal compartment. These lysosomal proteins are tagged with a unique marker: the mannose-6-phosphate (M6P) group, which is added exclusively to the N-linked oligosaccharides of lysosomal soluble hydrolases, as they pass through the cis-Golgi network. The M6P groups are then recognized by two independent transmembrane M6P receptors, present in the trans-Golgi network: the cation-independent M6P receptor and/or the cation-dependent M6P receptor. These proteins

bind to lysosomal hydrolases on the lumenal side of the membrane and to adaptins in assembling clathrin coats on the cytosolic side. In this way, the M6P receptors help package the hydrolases into vesicles that bud from the trans-Golgi network to deliver their content to endosomes that ultimately will develop into mature lysosomes, where hydrolases may start digesting the endocyted material[10].

Protein folding and N-glycosylation
In the ER, carbohydrates are added primarily to newly synthesized unfolded proteins. As a result, cells can use glycosylation to promote and regulate protein folding and quality control. Protein folding is a highly reliable process and is evolutionarily conserved[11]. Nonetheless, a small proportion of proteins misfold and the accumulation of aberrant proteins can be toxic. Proteins that fail to fold must be retained and recognized by the ER-associated degradation (ERAD) mechanism for turnover[12]. The first steps in glycan-directed protein folding are trimming of 2 terminal glucose residues from the A-branch of the glycan. The lectin-like ER proteins calnexin and calreticul bind to the remaining glucose residue in the $Glc_1Man_9GlcNAc_2$ structure. Calnexin is a type I integral membrane protein. Its lectin domain binds to the glycan and to the A-branch of the N-glycan that interacts with chaperones and with other parts of the substrate molecule. Calreticulin is the soluble homologue of calnexin, and is retained in the ER. This is a transient interactions, that allows the cleavage of the last glucose by glucosidase II, thereby preventing re-engagement of calnexin[13]. The UDP-glucose:glycoprotein glucosyltransferase (UGGT) inspects the released proteins and those still bearing unfolded domains are re-glucosylated by UGGT restoring the calnexin-binding site for another round of binding and release[12]. This process continues until the protein is fully folded or until it is degraded at the proteasome[14]. Well folded proteins all carry a $GlcNAc_2 Man_{8-9}$ structure.

Processing of the oligosaccharide
The structure remaining is subject to the action of a series of mannosidases that remove mannose residues in alfa 1-2 linkage (Figure 3). These enzymatic actions occur in the Golgi appartus. Some glycans will be processed to more complicated structures[1,9]. These are built on a common core that consists of just three mannose residues and two GlcNAc residues attached to the glycoprotein. This process is initiated by the attachment of a GlcNAc residue to the 1-3 arm of the core while it still contains five mannose residues. GlcNAc –transferase I initiates the re-elongation followed by a removal of two additional mannose residues from the 1-6 arm of the core by mannosidases.

Bi-antennary glycans are most abundant, but tri-antennary and tetra-antennary glycans are also common. The branched structures are typically extended by the addition of a single galactose and

sialic acid residue to each GlcNAc residue. Building steps for these structures are catalysed by galactosyltransferase and sialyltransferases[7].

It cannot be stressed enough that the exact glycan structure is defined by strictly regulated enzymatic processes that drive glycosylation. In contrast to glycation, the non-enzymatically driven binding of glucose to for example HBa1c, which is used in diabetes monitoring, glycosylation is dependent on the substrate itself (*in casu* a protein) and the enzymes, whose expression is strictly regulated. Each cell at a certain time or in a certain condition expresses a specific set of enzymes and thus glycans.
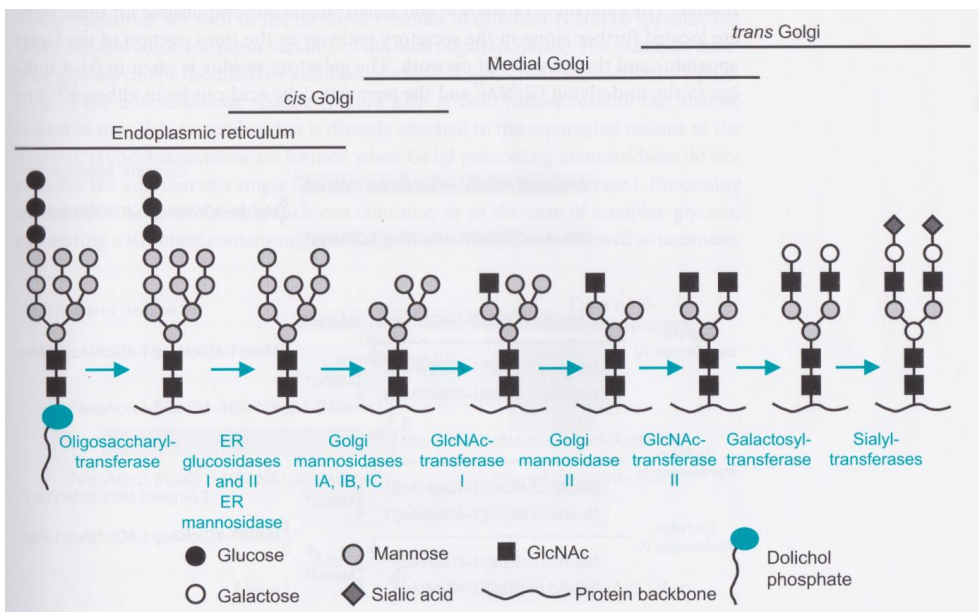
Figure 3. Processing of an initial high mannose N-linked glycan to generate a complex glycan.
(Source : Taylor and Drickamer. Introduction to Glycobiology. Oxford 2011).

Subdivision of N Glycans

The final result of N-glycan formation can be divided in high mannose, hybrid and complex type glycans (Figure 4). Complex type glycans are the most common type for secreted proteins.
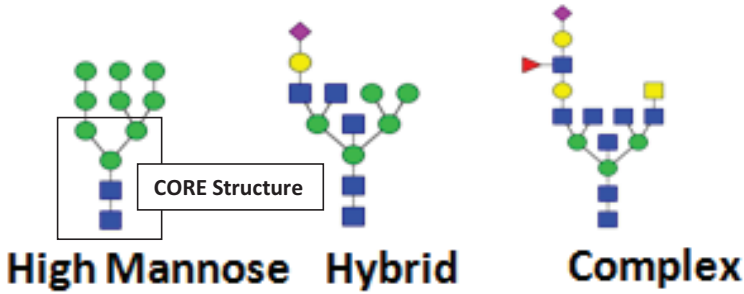
Figure 4. N-glycans can be divided into high mannose type glycans, hybrid type glycans and complex type glycans. All share a common core structure. Square : beta-linked GLcNAc; Green circle : mannose; yellow circle : galactose; red triangle: fucose; purple diamond: sialic acid

1.4.1.3 Glycomics

Glycomics is the comprehensive study of the glycome, which is the entire complement of sugars, whether free or present in more complex molecules of an organism. It includes genetic, physiological, pathological, and other aspects. It can also be defined as "the systematic study of all glycan structures of a given cell type or organism" and is a subset of glycobiology[715].

1.4.2 GLYCOMICS-BASED BIOMARKERS AND LIVER DISEASES : AN INTRODUCTION

Over the last decade the analysis of the glycome and the subsequently developed glycomics based biomarkers have received increasing attention and have evolved from an unknown rarity to a mature field of biomarker development. Glycomics is the "systematic study of all glycan structures of a given cell type or organism" and is a subset of glycobiology[16]. Protein glycosylation is regarded as one of the most common posttranslational modifications and is involved in 'specific recognition' events e.g. interaction between cells and the modulation and control of crucial biological processes e.g. protein folding[17].

N-Glycans are bound to proteins through an amide linkage to the Asparagine (Asn) (N) side chain in the sequence Asn-X-Serine/Threonin, where X is any amino acid except Proline, by a series of reactions catalyzed by a complex enzymatic machinery localized in the endoplasmic reticulum (ER) and Golgi compartments[18]. Alterations in N-linked glycosylation have been observed during the development of various diseases including cancer, infections and autoimmune diseases[19]. Emerging high-throughput (semi)-automated platforms have catalyzed the development of glycomics-based biomarkers that measure these posttranslational modifications.

1.4.2.1 Glycomics

Liver disease is a potential interesting field for serum glycomics-based biomarker discovery because the majority of N-glycans found in whole serum are attached to serum proteins produced in the liver[20] and to a lesser extent to immunoglobulins (Ig), being produced in B-cells[21]. Interestingly, consistent alterations in serum glycan structures have been described in liver diseases including viral hepatitis, fibrosis, cirrhosis and hepatocellular carcinoma[4].

For the last ten years, many potential glycomics-based biomarkers for various liver diseases have been developed. A first strategy is to measure alterations of the glycome on the total serum protein content[22], the second is to study glycoalterations on specific proteins, e.g. fucosylation of alpha foeto protein (AFP-L3)[23].

However, none of them have been adopted by clinicians for daily clinical use, due to lack of validation[24] and the use of expensive and labour-intensive technology, although most of these markers have a strong pathophysiological rationale. Furthermore, recently new techniques have been reported on more accessible platforms e.g. high throughput microfluid systems[25] or ELISA based techniques, which could accelerate clinical implementation.

Histologic assessment of liver tissue obtained by liver biopsy remains the gold standard for the diagnosis of liver diseases. However, liver biopsy has been challenged by non-invasive biological markers and by measurement of liver stiffness e.g. by transient elastrography[26]. Non invasive techniques are more comfortable for the patient and might overcome interobserver variability[27] and sampling error[28].

The goal of this review is to summarize the advances in the development of glycomics based markers and to identify biomarkers that have potential for clinical use.

1.4.2.2 Liver fibrosis and cirrhosis

Liver fibrosis is the result of excessive extracellular matrix deposition in the liver in response to chronic inflammatory injury and is determined by the replication balance between fibrogenesis and fibrosis degradation. When this balance favours fibrogenesis, there is a resulting accumulation of collagen and extracellular matrix, leading eventually to cirrhosis[29]. The gold standard for evaluation of fibrosis is a liver biopsy.

The whole serum N-glycome shows typical alterations in patients with liver fibrosis or cirrhosis. In 2004 Callewaert et al. described 8-aminopyrene-1,3,6-trisulfonic acid (APTS)-labeled (*N*-glycan) profiling on a high-throughput DNA sequencer (DNA sequencer-assisted fluorophore-assisted capillary electrophoresis or DSA-FACE)[3020] to generate profiles of serum protein N-glycans. In a typical desialylated serum protein N-glycan profile, 14 glycans were detectable and identified. A biomarker was developed named GlycoCirrhoTest[20] that distinguished compensated cirrhotic from non-cirrhotic chronic liver disease patients, with 79% sensitivity and 86% specificity. GlycoCirrhoTest is based on the increase in the proportion of bisecting GlcNac containing N-Glycans and a decrease in the proportion of triantennary N-Glycans. Diagnostic capacity was comparable to Fibrotest. Combining GlycoCirrhoTest with Fibrotest resulted in an increased diagnostic accuracy for compensated cirrhosis to 100% specificity and 75% sensitivity[20]. These glycoalterations were unrelated to etiology of liver disease[31]. Since 2010 sample preparation has been simplified and can be used on cheap high-throughput microfluidics CE platforms including the MCE-202 MultiNA, 2100 Bioanalyzer and eGene system[32].

The same technique was applied for the development of a marker for progressive fibrosis in chronic HCV[33] and chronic HBV patients[3435], called GlycoFibroTest, based on a gradual increase of the proportion of undergalactosylated bisecting GlcNac containing core fucosylated N-Glycans and a

decrease in the proportion of triantennary N-Glycans[33]. IgG depletion of sera[33], demonstrated that the undergalactosylation in the whole serum N-glycome is caused by undergalactosylation of immunoglobulins and not by liver derived proteins. Mehta et al.[21] also support the hypothesis that N-glycome changes in patients with fibrosis are partially derived from immunoglobulins. They observed alterations in glycosylation of an IgG reactive to galactose with increasing levels of fibrosis. Again, the main alterations were alpha1,6-core fucosylation and the increase of a bisected fucosylated biantennary N-glycan[21]. Sensitivity and specificity were more than 80%.

In contrast to these fibrosis markers, both glycan structures that form the GlycoCirrhoTest are not IgG related[20]. Thus, the GlycoCirrhoTest, that is highly specific for cirrhosis but not for increasing stages of fibrosis is based on glycans on liver secreted proteins and not changes in glycosylation of IgG.

In chronic HBV infected patients with various degrees of fibrosis, quantitative glycome profiling using Maldi-Tof MS identified 4 N-glycans that were incorporated into the Fibro-Glyco index[36]. Three of them were core-fucosylated or showed a bisecting GlcNac. Area under the curve (AUC) for detection of fibrosis (Ishak $\geq$3) was 0.912 (Sensitivity 85%; Specificity 84%) and for cirrhosis 0.911 (Sensitivity 88%; Specificity 83%).

All these biomarkers measure glycomic changes in the total serum protein content. Other biomarkers are based on the glycosylation of targeted serum proteins.

Glyco-alterations including fucosylation and desialylation on the acute-phase protein alpha1-acid glycoprotein (AGP) occur during progression of liver fibrosis. Fucosylation appears upon development of cirrhosis[37]. Kuno et al. developed a multiple lectin-antibody sandwich assay to study glyco-alterations in serum AGP. A combinations of 3 lectins, *Aspergillus oryzae* lectin (AOL), *Maackia amurensis* lectin (MAL) and *Datura Stramonium* agglutinin (DSA) was distinctive for the detection of severe fibrosis and cirrhosis[38]. This was followed by the development of an automated triplex lectin-antibody sandwich immunoassay assisted by an automated protein purification system (ED-01) and a bedside clinical chemistry analyzer (HISCL) for the acquisition of two glyco-parameters reacting against *Aspergillus oryzae* lectin (AOL) and **M***aackia amurensis* lectin (MAL)[39]. This LecT-Hepa test correlated with fibrosis stage (R=0,68) and showed an AUC of 0,86 for the diagnosis of severe fibrosis. These findings were confirmed in a multicenter study with an AUC of 0,882 for significant fibrosis[40].

Another lectin immunoassay, FastLec Hepa, measures quantitative and qualitative alterations of serum 90 K/Mac-2 binding protein (M2BP) using the *Wisteria floribunda* agglutinin (WFA). WFA[+]-

M2BP detects hyperfucosylation[41]. Again, diagnostic performance showed an AUC of more than 0,8 for significant fibrosis. These data were confirmed recently in a heterogeneous population[42].
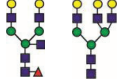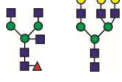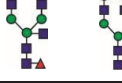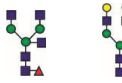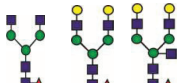
Colony stimulating factor 1 receptor (CSF1R) also appears to increase in sera of cirrhotic patients. An antibody-lectin sandwich ELISA was developed for detection of Wisteria floribunda agglutinin-reactive CSF1R (WFA⁺- CSF1R)[43]. Interestingly, results for WFA⁺-M2BP and WFA⁺- CSF1R were concordant (R=0,59). CSF1R and M2BP were both expressed in hepatic parenchymal cells and macrophages in regenerating nodules.

Recently, a sandwich ELISA assay was reported measuring fucosylated fetuin A. In patients with chronic HBV, decreasing levels of fucosylated fetuin A levels, a negative acute phase protein synthesized in hepatocytes, were described in respectively cirrhosis, HCC and healthy controls. However, an important overlap was present between cirrhosis and HCC patients[44].

In conclusion, the development of fibrosis and cirrhosis results in typical glycoalterations. Fibrosis is an inflammatory driven process with progressive liver damage, which is highlighted by the undergalactosylation of IgG related glycans. In established cirrhosis, altered glycan expression is primarily hepatocyte driven, as is reflected by the GlycoCirrhoTest[20]. The GlycoCirrhoTest defining glycan is a bisecting GlcNac, which is the product of N-acetylglucosaminyltransferase III (GnT-III)[45]. This enzyme is not expressed in hepatocytes in physiological conditions, but is expressed in hepatocytes in the regenerating liver[45]. Furthermore, GnT-III activity is increased in sera and liver tissue of cirrhotic patients[46,47]. As mentioned before, GCT is increased in cirrhotic patients, but not in patients with progressive stages of liver fibrosis, which supports the hypothesis that GCT increase is related to upregulation of GnT-III in regenerative nodules, which are the histological hallmark of liver cirrhosis and are not present in earlier stages of liver fibrosis.

In liver fibrosis repeated monitoring in order to assess fibrosis progression or response to therapy is an attractive strategy for the assessment of disease prognosis. Clinicians should keep in mind that biomarkers are expressed as continuous variables in contrast to histologic fibrosis staging systems (eg. Metavir), that turn a continuous variable into a categorical variable. Comparison between the new biomarkers and the histologic gold standard inevitably results in a large overlap between the different categories. However, this should be seen as a strength of the biomarker, and not as a weakness. This was nicely illustrated by the HALT-C trial study group, who studied computer-assisted morphometry in order to provide precise Histologic measurement of hepatic fibrosis on a continuous scale[48].

Table 1. Glycomics based biomarkers for liver fibrosis and cirrhosis. All patients had histologic grading of liver disease unless mentioned otherwise. Legend: AGP : alpha1-acid glycoprotein

Population	Number of patients	Technique	Diagnostic Glycan	Diagnostic performance (Fibrosis level Metavir)		Reference
				>=F2	F4	
Chronic liver disease	F0-F3: n = 52 F4: n = 48	DSA-FACE	**GlycoCirrhoTest** ↑NA2FB, ↓NA3		AUC 0,87	Callewaert et al.[20]
Chronic HCV	N = 400	DSA-FACE	**GlycoFibroTest** ↑NGA2FB,↓NA3	F2-F4: AUC 0.71		Vanderschaeghe et al. [33]
Chronic HBV	N = 173 Ishak 0-2 : 87 Ishak 3-6: 86	DSA-FACE	**GlycoFibroTest** ↑NGA2FB,↓NA3		AUC 0,710	Gui et al.[34]
Chronic HBV	N = 128	DSA-FACE	↑NGA2FB, ↓NA3	AUC 0,736	AUC 0,754	Qu et al.[35]
Chronic HBV	N = 46 Ishak 1 : 10 Ishak 2 : 9 Ishak 3-4 : 10 Ishak 5 : 8 Ishak 6 : 9	Maldi-Tof MS	**Fibro-Glyco Index** Increase of 2 core-flucosylated glycans and 1 penta-antennary glycan Decrease of NA2.	AUC 0.912	AUC 0.911	Kam et al. [36]
Chronic HCV Non HCV cirrhosis HCC	Ishak 1-2 :24 Ishak 3-5 :1 9 Ishak 6 : 57 N = 34 N=87	Lectin FLISA	↑Anti Alpha-Gal epitope on IgG Based on ↑serum glycans NGA2F NA2F NA2FB	AUC 0.900	AUC 0.930	Mehta et al.[21]

Chronic HBV and HCV	N=125 F0-1:33 F2: 32 F3: 31 F4: 29	Multilectin assay (12 lectins)	Cirrhosis : Increased α-1,3 fucosylation of the Lewis X antigen on AGP	AUC 0.760	AUC 0.900	Kuno et al.[38]
Chronic HCV	N = 175	Lectin Antibody immunoassay	**LectHepa** Increased α-1,3 fucosylation of the Lewis X antigen on AGP	AUC 0.730	AUC 0.950	Kuno et al.[39]
Chronic HCV	N = 183	Lectin Antibody immunoassay	**LectHepa** Increased α-1,3 fucosylation of the Lewis X antigen on AGP	AUC 0.802	AUC 0.929	Ito et al.[40]
Chronic HCV	N=209 F0–F1 : n=82 F2 : n = 52 F3 : n = 40 F4 : n = 35	Automated chemiluminescence enzyme immunoassay (HISCL)	**FastLec-Hepa** Increased α-1,3 fucosylation of the Lewis X antigen on AGP.	Fibrosis ≥ F2 AUC 0.797	AUC 0.910	Kuno et al.[41]
Chronic HCV (n=160) and HBV (n=21) Alcohol (n=12)	N=160 F0–F1 : n=82 F2 : n = 52 F3 : n = 40 F4 : n = 35	Automated chemiluminescence enzyme immunoassay (HISCL)	**FastLec-Hepa** Increased α-1,3 fucosylation of the Lewis X antigen on AGP.	AUC 0.812	AUC 0.795	Toshima et al.[42]
Fibrotest	Meta-analysis (HCV, HBV, alcohol, NASH)			AUC 0.66	AUC 0.69	Poynard et al.[50]
FIB-4 HCV (n=592)	F0 : n= 73 F1: n= 470 F2: n = 185 F3 : n = 85 F4 : n = 61		Age, platelets, ALT, AST	≥F3: AUC 0.85	AUC 0.91	Vallet-Pichard et al.[51]

Glycomics based test for fibrosis and cirrhosis are based on whole serum or specific serum proteins and show a diagnostic accuracy of at least 80% for advanced fibrosis (F3-F4), which is comparable to a commercialized test like Fibrotest. The main issue is the lack of external validation, with exception of the FastLec-Hepa test. In the validation cohort however, the diagnostic performance did not reach 0,80[49].

The question remains whether glycomics-based biomarkers for liver fibrosis and liver cirrhosis could challenge currently used biomarkers. Recently published EASL Clinical Practice Guidelines Non-invasive tests for evaluation of liver disease severity and prognosis[26] do not mention any glycomics-based biomarkers. The proposed biomarkers by EASL show a good diagnostic accuracy (AUC > 0.80%) for cirrhosis but are far less robust for detection of significant fibrosis (>= F2). FibroTest® and APRI are the most widely used and validated tests for fibrosis/cirrhosis and are being integrated in clinical practice. According to our overview (table 1), glycomics-based biomarkers might challenge FibroTest and APRI for detection of cirrhosis. Furthermore, a combination of FibroTest and GlycoCirrhoTest showed an increased performance for the diagnosis of cirrhosis[20]. However, confirmation in larger trials is necessary.

1.4.2.3 NAFLD and NASH

Non alcoholic fattly liver disease (NAFLD) encompasses the entire spectrum of fatty liver disease in individuals without significant alcohol consumption, ranging from fatty liver to steatohepatitis (NASH) and cirrhosis[52]. The burden of NASH is increasing and NASH has become the second leading cause for liver transplantation in the USA[53]. There is an urgent need for biomarkers that can distinguish NAFLD from NASH[54], as a liver biopsy is still required[55]. Hence, this ideal biomarker could identify NASH patients among NAFLD patients, a subgroup that needs a more careful follow up regarding the increased risk for progression to fibrosis[54].

The glycomic approach has generated interesting answers to this question (Table 2). In a first study the glycomic analysis using DSA-FACE according to Callewaert et al.[20] was applied in sera of 47 NAFLD patients including 82% NASH patients. Desialylated sera of NASH patients contained significantly higher relative abundance of NGA2F (a core fucosylated agalactosylated biantennary glycan) and lower relative abundance of NA2 (a galactosylated non fucosylated biantennary glycan). The logarithmic transformation of this proportion was called the GlycoNashTest and could identify NASH patients among NAFLD patients with an accuracy of 74,3%[56]. In contrast to earlier studies, the authors did not observe an increase of bisecting GlcNac carrying biantennary glycans in NASH

patients with fibrosis, as we observed in patients with chronic liver disease (viral hepatitis and alcoholic liver diseases) with increasing levels of fibrosis[22,33].
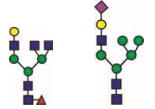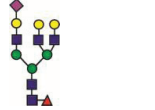
The GlycoNashTest was validated in a multicentre Belgian study of 275 obese patients undergoing bariatric surgery[57]. The AUC to distinguish NASH from borderline NASH and steatosis varied between 0,66 and 0,75, which was equal to K18F. Interestingly, the value of the GlycoNashTest increased significantly according to the level of lobular inflammation but not to the fibrosis stage. GlycoNashTest showed the lowest p-value[57]. The N-glycan profile of IgG that was isolated from sera of these patients confirmed the increased undergalactosylation in NASH patients. The diagnostic utility of GlycoNashTest was also demonstrated in a pediatric population[58]. Considering that the hallmark of NASH is the presence of an inflammatory infiltrate, it makes sense that the observed glycoalteration in NASH is immunoglobulin driven.

A Japanese group developed a so called "Sweetblot" technique, an automated protocol for integrated glycoblotting and MS[59]. In contrast to Callewaert et al[20], these glycans are not desialylated before analysis. In NASH patients, among 41 glycans examined, 8 glycans were increasingly expressed, 2 hybrid type, 3 bisected biantennary type and 2 triantennary type glycans. Among those, 3 glycans m/z 1955 (a bisected biantennary type fucosylated glycan), m/z 2032 (a hybrid type glycan) and m/z 2584 (a triantennary fucosylated glycan) showed an UAC above 0,83[60]. These glycans showed a positive correlation with the level of lobular inflammation, ballooning and steatosis.

The glycoalteration of specific glycoprotein has proven helpful in the diagnosis of NASH. Measured by a lectin-antibody ELISA kit, serum Fucosylated Haptoglobin (Fuc-Hpt) levels have been reported as a marker for NASH (AUC 0,734)[61]. Fuc-Hpt levels showed a significant increase according to increasing hepatocyte ballooning score. In almost 900 NAFLD patients (without histologic grading of liver disease) serum Fuc-Hpt increased stepwise with increasing FIB-4 index[61]. In the mean time, the same group developed an ELISA assay based on glycoalterations in Fuc-Hpt and Mac2BP. The latter was initially developed as fibrosis marker. The combination of both yielded a diagnostic performance for distinguishing NAFLD from NASH of 0,854 in a pilot cohort and 0,844 in a large validation cohort[62]. As reported earlier, Fuc-Hpt reflects hepatocyte ballooning and Mac2BP detects the presence of fibrosis.

The current gold standard for diagnosis for NASH remains liver biopsy. A promising alternative would be the measurement of cytokeratin-18 (CK-18) that showed an AUC of 0.711 to distinguish simple steatosis from NASH[63]. However, subsequent cohorts showed disappointing diagnostic values (AUC of 0.631 and 0.500)[64]. In our analysis, the glycan based GlycoNashTest showed a higher diagnostic accuracy than CK-18[57].

Table 2 : Glycomics based biomarkers for NAFLD and NASH. All patients had histologic grading of liver disease unless mentioned otherwise.

Population	Number of patients	Technique	Diagnostic Glycan	Diagnostic performance	Reference
NAFLD and NASH	NAFLD : n=9 NASH : n = 38	DSA-FACE	GlycoNashTest ↑NGA2F,↓NA2	NASH : AUC 0.743	Chen et al.[56]
NAFLD and NASH	NAFLD : n=199 NASH : n=76	DSA-FACE	GlycoNashTest ↑NGA2F,↓NA2	NASH : AUC 0.66 – 0.75	Blomme et al.[57]
Paediatric NAFLD and NASH	NAFLD : n=5 NASH : n=46	DSA-FACE	GlycoNashTest ↑NGA2F,↓NA2	NASH : AUC 0.72	Blomme et al.[58]
NAFLD and NASH	NAFLD : n=15 NASH : n=42	Sweetblot (integrated glycoblotting and MS)	m/z 1955 m/z 2032 m/z 2584	NASH : AUC 0,833 0,863 0,866	Yamasaki et al.[60]
NAFLD and NASH	NAFLD : n= 19 NASH : n= 107	Lectin Ab ELISA	Fucosylated Haptoglobin	NASH : AUC 0,734	Kamada et al.[61]
NAFLD and NASH	**Training cohort** NAFLD : n= 29 NASH : n= 95 **Validation cohort** NAFLD : n= 169 NASH : n= 213	Lectin Ab ELISA	Fucosylated Haptoglobin and Mac2bp	AUC : 0,854	Kamada et al.[62]

In conclusion, a biomarker to identify NASH patients among NAFLD patients is an unmet clinical need. Glycomic changes in whole serum or on targeted glycoprotein (eg. Fuc-Hpt and Mac2BP) are promising markers for NASH.

1.4.2.4 Hepatocellular carcinoma
Diagnostic biomarkers

Alterations in glycosylation of glycoproteins has been reported in several cancers, e.g. ovarian cancer[65], colorectal cancer[66] and prostate cancer[67] amongst others. They are involved in the entire spectrum of carcinogenesis, from tumor progression, tumor cell differentiation, over cell- cell interaction and tumor cell adhesion to metastasis[68]. For example, the histoblood group Lewis (Le) antigens are found in most human epithelial tissues attached to glycolipids and glycoproteins. The expression of the sialylated antigens, SLe[a] (a branch alfa-1,4-fucosylated tetrassacharide) and SLe[x] (a branch alfa-1,3-fucosylated tetrassacharide) is significantly enhanced in cancer. Both SLe[a] and SLe[x] contribute to hematogenous metastasis, in which blood-invading cancer cells adhere to blood vessels endothelial cells in requiring the presence of carbohydrate ligands on cancer cells and at the same time E-selectin receptors on endothelial cells. SLe[a] mediates adhesion of cancer cells derived from the lower digestive organs (colon and rectum), pancreas and biliary tract, while the SLe[x] mediates adhesion of breast, ovarian and pulmonary cancer cells[69].

HCC has been the first focus of glycobiomarker development. Similar glycoalterations in serum and in HCC tissue suggest that glycans are involved in tumor formation and development[70]. For decades alfafoetoprotein (AFP) has been used as a diagnostic marker for HCC and it remains the best diagnostic and prognostic biomarker for clinical use, despite the overall disappointing accuracy[71]. AFP is a glycoprotein produced by the yolk sac and the fetal liver[45]. AFP has a single N-linked oligosaccharide with a biantennary complex-type structure. During cancer development altered terminal sialylation and core fucosylation appear. This fucosylation is detectable using *Lens culinaris* agglutinin (LCA) lectin and the LCA-reactive fraction of AFP (AFP-L3) is an improved diagnostic accuracy for HCC compared to AFP levels[72]. Using a cut off of 10 ng/ml, sensitivity for detection of (early) HCC was 60% and specificity 90%[73].

The technique developed by Callewaert[20] for high-throughput analysis of N-glycans was also applied on HCC patients. In HBV-related HCC patients NA3Fb, a branch alfa-1,3-fucosylated triantennary glycan (involving the SLe[x] epitope) was more abundant than in patients with cirrhosis or fibrosis without HCC. On the other hand the abundance of a bisecting core alpha-1,6-focusylated biantennary glycan (NA2FB) was lowered[74]. The logarithmic transformation of this ratio was renamed

GlycoHCCTest and reached a diagnostic accuracy of 81%, equal to AFP in this cohort and showed a clear correlation with tumor stage. A Chinese group applied the same technique in HBV related HCC patients and reported that in an increase of NA3Fb, an alfa-1,3-fucosylated triantennary glycan and a decrease of NG1A2F, an agalacto monogalactosylated core-1,6-fucosylated biantennary glycan, was associated with HCC with a diagnostic accuracy of 0,873[75]. Furthermore, this glycomarker compared to AFP showed more diagnostic accuracy for the presence of vascular invasion.

An integrated platform using "glycoblotting" and MS was developed for N-glycan profiling of whole serum[76]. This group claims a 100% diagnostic accuracy for HCC using a subset of 4 glycans (Man5, NGA2, NA2G1 and NA2). Interestingly, none of these glycans are fucosylated.

In a set of 83 N-glycans in human serum, 53 glycans were altered in HCC patients compared to a control population[77]. In a multivariate logistic regression, 3 glycans remained significantly associated with HCC. The combination of these glycans in a blinded validation set showed an AUC of 0,960. An increase was seen in the abundance of a biantennary bisecting glycan and a decrease in triantennary and tetra-antennary complex glycans. None of these glycans were fucosylated. In contrast, in a exploratory analysis of 27 HCC patients, HCC patients could be identified with a high diagnostic accuracy (AUC 0.811) due to increased fucosylation of 5 serum proteins (C3, CE, HRG, CD14 and HGF)[78].
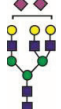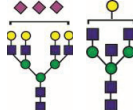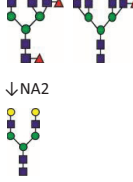
Using LC-ESI-MS, compared to cirrhotic patients without HCC, 8 N-glycans were upregulated and 3 N-glycans were downregulated in HCC patients[79]. These glycans could be grouped in 4 clusters: a cluster of 3 biantennary glycans (upregulated), a cluster of 4 beta-1,6,GlcNAc branching glycans (upregulated), a cluster of 5 other beta-1,6,GlcNAc branching glycans (downregulated) and a cluster of 5 tetra-antennary glycans (upregulated). Cluster 3 and 4 are downstream products of GnT-V.
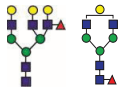
The Sweetblot technology, also used for NASH biomarkers, was applied on a larger cohort of 114 HCC patients on a background of viral hepatitis[80]. The ratio of m/z 3195, a tri-sialylated triantennary complex type glycan with fucose residue on m/z1914 (a bigalactosylated biantennary fucosylated glycan) showed an AUC of 0,810 for the distinction of HCC from liver cirrhosis and chronic viral hepatitis[80]. Both glycans were similar to those represented by the GlycoHCCTest[74].

Beside these whole serum protein content glycomic fingerprinting, glycoalterations on specific serum proteins in HCC were also studied.

Debruyne et al.[81] isolated Hemopexin, a liver secreted protein, from human serum. Specific glycomic patterns on Hemopexin showed increased branching α-1,3-fucosylated multi-antennary glycans in HCC. A Chinese group reported an increase of fucosylation and sialylation of paraoxonase 1

Table 3 : Glycomics based biomarkers for HCC. All patients had histologic grading of liver disease unless mentioned otherwise. Exploratory studies with low number of patients and/or lack of HCC size were excluded from the table.

Population	Number of patients	HCC size	Technique	Diagnostic Glycan	Diagnostic performance	Reference
Diagnostic Biomarkers						
HCC in Chronic HBV	N= 227	TNM classification : T1 : n=6 T2 : n= 28 T3 : n=59 T4 : n=5	DSA-FACE	GlycoHCCTest ↑NA3FB ↓NA2FB	AUC : 0.81 Significant correlation with TNM stage	Liu et al.[74]
HCC in chronic HCV	N=73	Stage I/II: n=18 Stage III/IV: 33 (AJCC staging)	MALDI-TOF MS	Increased abundance Decreased abundance	AUC 0.960	Goldman et al.[77]
HCC	N=20	Not reported	DSA-FACE	Hemopexin Glycan marker: ↑NA3Fcb ↑NA4Fb ↓NA2	AUC 0.920	Debruyne et al.[81]

HCC	N=27	Very early HCC	Lectin Antibody Array	Increased fucosylation in C3, CE, HRG, CD14 and HGF	AUC 0.811	Liu et al.[78]
HCC in Chronic HBV	N=145	Stage I: n=67 Stage II/III/IV: n=69 (AJCC staging)	DSA-FACE	↑NA3Fb↓NG1A2F	AUC 0.873	Fang et al.[75]
HCC	N=27	TNM 1/2	Tandem lectin affinity chropmatography	↑fucosylation and sialylation of serum PON1	AUC 0.902	Sun et al.[82]
HCC	N=110	Not reported	Lectin ELISA	↑fucosylation serum PON1	AUC 0.803	Zhang et al.[84]
HCC (chronic HBV/HCV/ Alcohol)	N=50 (18/42/40)	TNM: Stage I : *n* = 7 Stage II: n=24 Stage III: n=11 StageIV:n= 8	MALDI-QIT-TOF	Increased Bifucosylation of Haptoglobin	AUC 0.821-0.843	Zhu et al.[87]

heteroplasmon (PON1) in sera of early HCC patients[82] (AUC 0.902). PON1 is a calcium-dependent hydrolase protein synthesized mainly by liver cells and secreted in the circulation and contains 3 identified glycosylation sites[83]. Recently, an ELISA was reported for measuring fucosylation index of PON1. AUC for the diagnosis of early HCC was 0.803[84].

Analysis of site specific glycans on the serum haptoglobin beta chain revealed increased sialylation on the NLFN[207]HSEN[211]ATAK site and an overall increased fucosylation[85]. Another study reported increased fucosylation on plasma haptoglobin and vitronectin (VTN)[86]. Quantitative analysis of serum haptoglobin fucosylation unequivocally showed that the presence of core and branch fucosylation

could differentiate between HCC and cirrhosis (AUC > 0.80)[87]. In HBV and alcohol related liver but not in HCV related liver disease, total fucosylation index was also discriminative for HCC. This finding however should be interpreted with caution considering the limited number of patients in every subgroup.

In summary, consistent alterations of glycosylation have been observed in HCC : increased fucosylation, increased presence of bisecting GlcNac residues and increased branching leading to the accumulation of triantennary and tetra-antennary glycans[4]. These processes are the result of the upregulation of fucosyltransferases and the N-acetylglucosaminyltransferases III and V[4,88,89]. Targeting glycoalterations on specific proteins is not superior to whole serum protein N-glycan analysis for HCC diagnosis[90].

1.4.3.5 Discussion

Biomarkers are playing an increasingly important role in the management of patients with chronic liver diseases. The last decade, we witnessed an exponential increase in glycomics-based biomarkers in the field of hepatology. This review summarized data regarding glycobiomarkers for liver fibrosis/cirrhosis, NAFLD/NASH and HCC.

The first observation is the important number of studies regarding glycobiomarkers by several groups using different techniques. Diagnostic markers were developed for fibrosis/cirrhosis, NAFLD/NASH and HCC with a good diagnostic performance (AUC >0,8). Unfortunately, most biomarkers lack external validation. However, consistent glyco-alterations were observed according to the underlying condition. We expect validation studies in the upcoming years.

Second, the observed glyco-alterations are supported by a pathophysiological rationale, eg. the increased presence of fucosylatransferases in HCC, reflected by the increased fucosylation of several glycoproteins[91,61,92,84,88]. Three glycosyltransferases deserve further attention: N-acetylglucsaminyltransferase V (GnT-V), N-acetylglucosaminyltransferase III (GnT-III) and alfa,1-6 fucosyltransferase (alfa,1-6FT). GnT-V catalyzes beta1-6 branching of N-acetylglucosamine on asparagines (N)-linked oligosaccharides of cell proteins, GnT-III catalyzes the formation of the bisecting GlcNac in a beta 1,4 linkage [45,47,93] and alfa,1-6,FT moves a fucose residue from GDP-fucose to N-linked type complex glycopeptides with an alfa,1-6 linkage[94]. Both GnT-III and GnT-V are increased in HCC and compete for the same substrate. In HCC, Beta1-6,GlcNac branching catalysed by GnT-V correlates with TNM classification of HCC[95] and with metastasis[96]. On the other hand, an increase of GnT-III might suppress GnT-V activity and subsequent GlcNac beta,1-6, branching, what

could counteract tumor progression[34,96,97], which was the rationale for the GlycoHCCTest[74] and confirmed by Fang et al[34].

Although hyperfucosylation has been reported in cirrhosis[21] and NASH[61], hyperfucosylation seems to be preferentially related to HCC[91]. The majority of glycomics based biomarkers for HCC are based on increased fucosylation, preferentially core fucosylation. Two markers are not based on an increase of fucosylation, although increased fucosylation was seen in HCC sera in one study[77] and not reported nor denied in the other study[76].

However, Methta et al. studied core fucosylation levels in HCC tissue and surrounding non-tumoral liver tissue. In 16 samples, fucosylation was not consistently increased in HCC tissue compared to adjacent tissue and tissue of healthy controls[98]. In contrast, increased levels of a tetra-antennary glycan (A4F4) were observed in HCC tissue[98]. In this study this same glycan was also statistically increased in a small set of 18 HCC sera compared to serum of 9 cirrhotic patients.

The third observation is that these glycoalteration can be studied on whole serum proteins or on isolated proteins eg. haptoglobin, PON1, fibronectin etc. Diagnostic performance is not increased by targeting single proteins. Clinical application requires a rapid high throughput automatised technique, what could favor whole serum analysis. Several markers could fulfill this requirement including the GlycoHepatoTest (including the GlycoCirrhoTest[20], GlycoFibroTest[25], GlycoNashTest[57] and GlycoHCCTest[74]) in a single analysis and the Sweetblot[59] technique.

If we take a closer look at the specific domains where these biomarkers have been tested, interesting conclusions can be drawn regarding the application in clinical practice.

LIVER FIBROSIS AND CIRRHOSIS

The first observation is that increasing levels of fibrosis correlate with inflammatory changes. Our group[33] amongst others[90] formerly showed that fibrosis was associated with increasing levels of undergalactosylated IgG related glycans. Probably, these changes reflect the ongoing inflammation rather than the established fibrosis. A Japanese team developed the Fast-Lec Hepa, an automated immune-assay that measures hyperfucosylation on Mac 2 Binding protein using a lectin immunoassay. However, the underlying characteristics of the test have not been revealed[41]. All these markers show excellent correlation with fibrosis stage. Although these markers show excellent diagnostic accuracy (AUC > 0.900 for advanced fibrosis) these are not superior to other non glycomic serum fibrosis markers like fibrotest[99] or FIB4[51] that have now been adopted by clinicians.

A special test in this series is the GlycoCirrhoTest. It is not a marker of fibrosis but a specific marker of cirrhosis. In contrast to the GlycoFibroTest that measures inflammation, the GlycoCirrhoTest is defined by an increase of a bisecting GlcNAc, which is the product of GnT-III[20]. The activity of this enzyme is increased in cirrhotic nodules and absent in normal resting hepatocytes[45]. Given that the cirrhotic nodule is the hallmark of cirrhosis, this pathophysiological rationale supports the strong diagnostic performance for established compensated and decompensated cirrhosis with a high specificity (>90%) at a specificity of more than 60%.

NON-ALCOHOLIC FATTY LIVER DISEASE (NAFLD)

There is a real unmet medical need for non-invasive markers of NAFLD. For the differentiation between simple steatosis and NASH a liver biopsy is required. Specific glycomic signatures have been observed in patients with NASH compared to simple steatosis. The GlycoNASHTest used the same technology as the GlycoFibroTest and the GlycoCirrhoTest. The GlycoNASHTest is based on an increase of undergalactosylated glycans and correlates with lobular inflammation, ballooning and steatosis but not with fibrosis[57]. Although the AUC for differentiation between NASH and steatosis was adequate (AUC = 0.750), an important overlap was seen between both groups, hampering the use in clinical practice. A Japanese group developed an automated protocol for integrated glycoblotting and MS (*Sweetblot* technique) and defined another glycomic signature that correlated with lobular inflammation, ballooning and steatosis[60]. Increased fucosylation of Haptoglobin was also associated hepatocyte ballooning[62] in another study.

The field for biomarker development in NAFLD is huge. In Western countries it affects 17–46% of adults[100]. The current EASL guidelines on the management on NAFLD that have been published only a few months ago advocate that NASH "has to be diagnosed by a liver biopsy showing steatosis, hepatocyte ballooning and lobular inflammation (A1)"[100]. This illustrates the total lack of efficient biomarkers for the diagnosis of NASH. This is a real opportunity for research groups to refine and improve glycomics-based biomarkers for the diagnosis of NASH.

HEPATOCELLULAR CARCINOMA
The field of HCC shows the largest research in glycomics-based biomarkers. Glycomic alterations can be summarized as increased fucosylation, increased bisecting GlcNAc residues, and increased branching with complex tri- and tetra-antennary glycans.

In the evolution towards personalized medicine, the real medical need for HCC biomarkers is limited to (1) the very early diagnosis of HCC where cure is still possible and (2) as markers of response to therapy and relapse. This overview shows that most glycomics-based biomarkers have excellent

results in large established tumours, but have not adequately been tested in very small tumours. This proves that it makes sense to measure glycomic alterations in serum of HCC patients as these changes are reflected by an altered glycosylation machinery in HCC tumours. However, these findings need to be refined in early tumours in order to prove a real clinical benefit.

Interestingly, probably the best glycomic biomarker for HCC is probably the oldest: AFP-L3. AFP-L3 is the fucosylated L3 fraction to total AFP ratio. It is superior to classic AFP measurement for early diagnosis of HCC and it has been shown that pre-treatment AFP-L3 levels in HCC patients undergoing a treatment have a prognostic value[101]. The use of these marker is however not advocated by current guidelines[102].

In conclusion, glycomics are a sweet and promising field of biomarker development with robust biomarkers for several liver diseases. Although most markers have not been externally validated, consistent findings amongst different research groups and the well described pathophysiological rationale of these glycoalterations could shine a new light on glycobiomarkers that remain a hidden gem.

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II.   Study Goal

The introduction of this work reviews the current knowledge of glycomics-based biomarkers for the diagnosis of chronic liver disease. The goal of this PhD thesis was to explore new horizons in the application of glycomics-based biomarkers, with a focus on **prognostic markers** in liver disease and liver transplantation.

Indeed, in this era of personalized medicine, prognostic markers will gain tremendous importance in the development of the therapeutic strategy of a given patient.

The prognostic potential of glycomics-based biomarkers was explored in several areas of liver disease.

1. **The potential of <u>serum</u> glycomics-based biomarkers to predict the risk of hepatocellular carcinoma development in compensated cirrhosis.**

   Liver cirrhosis is the major risk factor for development of HCC. The current strategy used by clinicians is to screen cirrhotic patients using ultrasonography (with or without serum AFP measurement) every 6 months in order to detect early and treatable HCC lesions. We explored the role of the GlycoCirrhoTest, a non invasive serum marker, as a glycomarker to stratify between high- and low risk patients for the development of HCC.

   Potential clinical relevance : A specific glycomic signature (GlycoCirrhoTest) of cirrhotic patients with a high-risk for development of HCC could lead to the implementation of personalized surveillance protocols for patients, according to the GlycoCirrhoTest value. This could increase awareness among patients with a high risk for HCC development to adhere to screening programs and could reassure low-risk patients. Futhermore, it could prove cost-effective as the number of visits in a significant number of low-risk patients might be reduced.

2. **The potential of glycomics-based biomarkers to assess the quality of the donor liver graft before liver transplantation using <u>perfusate</u> analysis.**

   In liver transplantation, the quality of the donor liver graft has a major impact on the outcome of the patient after transplantation. The goal of this work was double:

(1) *Evaluation of the technical feasibility of glycomic analysis of perfusate*

We evaluated whether the method for N-glycan analysis in serum developed by Callewaert et al.[1] is applicable in perfusate, the preservation fluid in which the liver is transported, often for many hours, from donor to recipient.

(2) *Development of glycomic-based biomarkers for primary non function after liver transplantation.*

We explored the role of the glycomics signature in perfusate of different outcomes after liver transplantation, including normal liver function, early allograft dysfunction and primary non function. In patients suffering from primary non function a specific and highly discriminative glycomic signature was discovered, leading to the development of a new biomarker.

A patent application PCT /EP2016/ 065383 was filed for this biomarker.

Potential clinical relevance : The identification of a specific glycomic signature in perfusate of patients with a high risk of PNF development enables transplant physicians to make evidence-based decisions in organ allocation. A high risk for PNF development can guide the removal of high-risk organs from the organ donor pool or reassure the transplant physician that the risk for PNF using this specific organ will be low.

3. **The potential of glycomics-based biomarkers to assess the outcome after liver transplantation in the early transplant period using <u>serum</u> analysis**

In this project we performed a sequential glycomic analysis of serum in the immediate peri-transplant period. The goal was to study the evolution of the serum glycome in the first 2 weeks after liver transplantation and to explore glycomic signatures predictive for survival of the liver graft and the patient.

Potential clinical relevance : A specific glycomic signature that identifies patients with a high risk of organ loss or even death during the first year after liver transplantation is a valuable tool for decision-making in the post-transplant period. It could allow a closer follow-up of these patients and could endorse the decision to perform a retransplantation in a particular patient with problematic liver function after LT and a high GlycoTransplantTest value.

4. **The potential of glycomics-based biomarkers to assess the appearance of acute cellular rejection after liver transplantation using a non-invasive** <u>**serum**</u> **analysis.**

Acute cellular rejection (ACR) is a frequent complication after liver transplantation and requires liver biopsy for diagnosis. The goal of this work was double:

(1) A *literature review* regarding potential non-invasive diagnostic markers for ACR was performed (included in the introductory section).

(2) The *potential of glycomics-based markers for the non-invasive diagnosis of ACR* was explored.

Potential clinical relevance : Reliable non-invasive markers for ACR are an unmet clinical need. If a specific glycomic signature could be found during the occurrence of ACR, it could lead to the development of a non-invasive biomarker for ACR and thus avoid liver biopsies in patients after liver transplantation.

5. **The potential of glycomics-based biomarkers to assess outcome in acute liver failure using** <u>**serum**</u> **analysis**

Acute liver failure is a serious liver condition occurring in otherwise healthy often young patients without underlying liver disease. In some patients urgent liver transplantation is required.

The goal of this work was to explore the dynamic glycomic changes in serum during the occurrence of acute liver failure. Furthermore, we provide a proof of concept that the glycomic signature at admission can predict transplant-free survival.

Potential clinical relevance : A glycomic signature that can predict transplant-free survival in ALF patients could allow to withhold patients from liver transplantation and guide only those patients to liver transplantation who need it in order to survive the ALF episode.

III. The potential of glycomics-based biomarkers to predict the risk of hepatocellular carcinoma development in compensated cirrhosis

A glycomics-based test predicts the development of hepatocellular carcinoma in cirrhosis

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3.1 ABSTRACT

Purpose: Cirrhosis is a major risk factor for the development of hepatocellular carcinoma (HCC), but remains underdiagnosed in the compensated stage. Fibrosis progression and cirrhosis are associated with changes in blood serum glycomic profiles. Previously, the serum glycomics-based GlycoCirrhoTest was shown to identify 50-70% of compensated cirrhosis cases in chronic liver disease cohorts, at >90% specificity. This study assessed GlycoCirrhoTest for the risk of HCC development in compensated cirrhosis.

Experimental Design: Serum glycomics were analysed in sera of 133 patients with compensated cirrhosis collected between 1995 and 2005 in a surveillance protocol for HCC using an optimized glycomic technology on a DNA sequencer.

Results: Baseline GlycoCirrhoTest values were significantly increased in patients who developed HCC after a median follow-up of 6.4 years as compared to patients who did not. For patients with a baseline GlycoCirrhoTest exceeding 0.2, the hazard ratio for HCC development over the entire study (Cox regression) was 5.1 (95% CI 2.2-11.7;p<0.001), and the hazard ratio for HCC development within 7 years was 12.1 (95% CI 2.8-51.6;p=0.01) based on cut-off value optimized in the same cohort. An absolute increase in GlycoCirrhoTest of 0.2 was associated with a hazard ratio of 10.29 (95% CI 3.37-31.432.11;p<0.001) for developing HCC. In comparison, the hazard ratio for the development of HCC within 7 years for AFP levels above the optimal cutoff in this study (5.75 ng/ml) was 4.65 (95% CI 1.588-13.607).

Conclusions: This prognostic study suggests that GlycoCirrhoTest is a serum biomarker that identifies compensated cirrhotic patients at risk for developing HCC. Screening strategies could be guided by a positive test on GlycoCirrhoTest.

3.2 INTRODUCTION

Hepatocellular carcinoma (HCC) represents up to 85% of the primary liver cancer burden[2]. In recent years, an increasing number of biomarkers have been proposed for the diagnosis of HCC[3]. Other markers have been proposed to better assess the prognosis of the outcome of HCC[4]. However, to increase the effectiveness of screening aimed at detecting HCC at the early stage that is amenable to curative therapy, it is important to accurately identify the main risk groups. In this regard, it is well established that liver cirrhosis is the most important risk factor for HCC development. Indeed, in
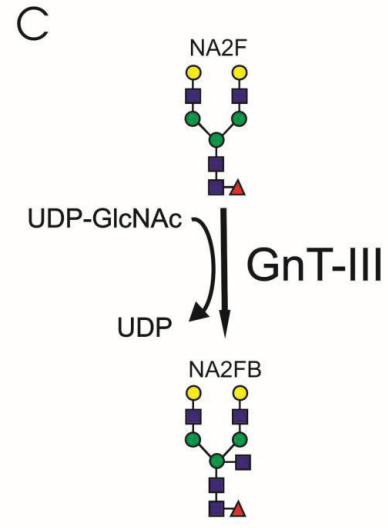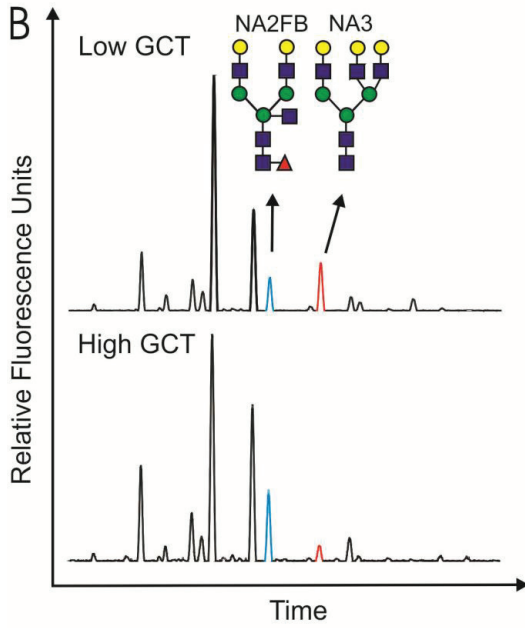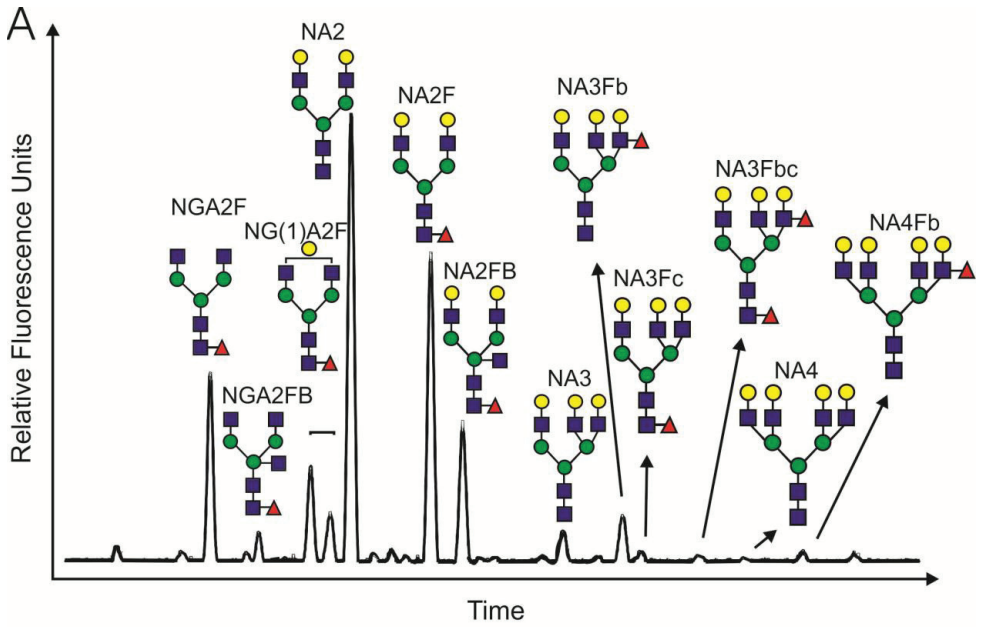
Figure 1 : The glycomic analysis and GlycoCirrhoTest

Panel A: 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 bisecting biantennary (NGA2FB), peak 3 and peak 4 are single monogalacto, 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 9 is the core-alpha-1,6-fucosylated triantennary glycan NA3Fc, 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.

Panel B: The GlycoCirrhoTest profile of patients with cirrhosis is characterized by an increase in the relative expression of NA2FB, a bisecting N-acetylglucosamine containing N-Glycan, and a decrease in the relative expression of NA3, a triantennary N-glycan on glycoproteins in serum. The upper glycomic profile shows a patient with a low GlycoCirrhoTest, who did not develop HCC during follow-up. The lower glycomic profile shows a patient with a high GlycoCirrhoTest, who did develop HCC during follow-up. In this patient, the relative expression of NA2FB is increased while the relative expression of NA3 is decreased.

Panel C: N-acetyl-glucosaminyltransferase III (GnT- III) catalyzes the addition of an N-acetylglucosamine (GlcNAc) residue from the uridinediphosphate (UDP)-GlcNAc donor to core-mannose in a β 1-4 configuration and forms bisecting GlcNAc.

most prevalent aetiologies of chronic liver disease, the vast majority of HCC cases originate on a background of cirrhosis[5678], likely because hepatocellular cell proliferation in the inflammatory context of cirrhotic nodules provides a strongly enlarged pool of dividing hepatocytes in which mutagenesis can result in tumour formation. In patients with compensated cirrhosis the annual incidence of HCC ranges from 1% to 8%[9]. EASL and AASLD guidelines advocate systematic ultrasound-based screening for HCC in any patient with cirrhosis on the basis of ultrasonography (US) every 6 months[1011]. The aim of screening is to detect small tumours with more chance of curative therapy[12]. This screening strategy in cirrhotic patients showed a reduction in HCC mortality rates[13] and it is cost-effective[14]. Unfortunately, the proportion of cirrhosis patients who do have screening remains low. For instance, in a North American cohort, less than 20% patients with HCC reported to have received regular screening before diagnosis[15]. An important reason for this is that the compensated stage of

liver cirrhosis remains underdetected. The current diagnosis of compensated cirrhosis is through liver biopsy in chronic liver disease patients. However, biopsy is unsuited for regular patient monitoring and a reliable and specific non-invasive biomarker that identifies the cirrhosis-characteristic hepatocyte proliferation that predisposes to HCC development could fill this gap.

We have previously shown that the GlycoCirrhoTest, a "glycomics" biomarker based on profiling of the N-glycans from the total serum protein using capillary electrophoresis (CE), could distinguish chronic liver disease patients with compensated cirrhosis from those with earlier stages of fibrosis. Furthermore, GlycoCirrhoTest has been optimized for use in clinical laboratories, using high-throughput DNA sequencers or CE-based analysers[16,17], including those that are in use in clinical chemistry for routine serum protein electrophoresis (unpublished results).

The GlycoCirrhoTest profile of patients with cirrhosis is characterized by an increase in the proportion of bisecting N-acetylglucosamine (GlcNAc)- containing N-glycans and a decrease in the proportion of triantennary N-glycans on glycoproteins in serum (Figure 1). The enzyme N-acetylglucosaminyltransferase III (GnT-III) catalyzes the addition of a bisecting GlcNAc residue in β1,4 linkage to the β-linked mannose of the trimannosyl core structure of N-linked oligosaccharides of glycoproteins, using UDP-GlcNAc as donor substrate[18]. This leads to the formation of the defining sugar moiety of the GlycoCirrhoTest (Figure 1). Neither bisecting GlcNAc residues nor GnT-III activity are detectable in a rat model in non-nodular liver tissue surrounding liver nodules or in livers of age and sex matched control rats[19,20]. On the other hand, in 2 different rat models of HCC (induced by 1,2-dimethyl-hydrazine or diethylnitrosamine) significant levels of GnT-III expression were observed in hepatic non-malignant cirrhotic nodules as well as in HCC nodules. Significantly increased GnT-III activity was also observed in sera and liver tissue of patients with nodular cirrhosis and HCC but not in patients with chronic hepatitis without cirrhosis[21,22]. After treatment of HCC with transarterial chemoembolization or percutaneous ablation, serum GnT-III levels decreased markedly[21,22]. Altogether, these data suggest that GnT-III is produced in the liver in (pre)neoplastic states but not in non-cirrhotic chronic liver diseases or normal liver tissue.

Because of this biology, in the present study we had the aim of investigating the hypothesis that a high GlycoCirrhoTest value may identify those compensated cirrhosis patients with the highest risk of progressing to HCC during follow-up. For comparison, we also studied the prognostic value of FIB-4[23] and alpha foetoprotein (AFP).

3.3 PATIENTS AND METHODS

3.3.1 STUDY COHORT

The study population consisted of 133 consecutive cirrhotic patients. Serum samples were prospectively collected between 1995 and 2005 at the Department of Hepatology of Beaujon Hospital (Clichy, France) and stored at -20°C. These patients were part of a large French multicentric prospective randomised trial on behalf of the "Groupe d'Etude et de traitement du carcinome hépatocellulaire" (GRETCH) that compared ultrasonographic surveillance of HCC in cirrhosis at 3- versus 6-month interval[24]. The study conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the Ghent University Hospital ethics committee.
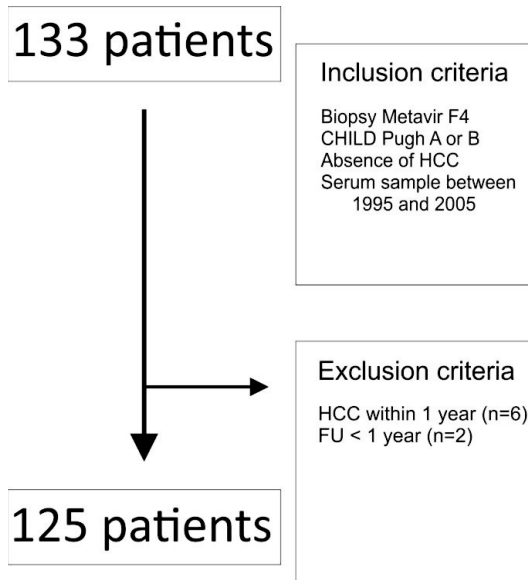
Figure 2: Flowchart with overview of inclusion- and exclusion criteria.

All patients had biopsy proven Child-Pugh A (n=116) or B (n=10) cirrhosis at the time of serum sampling. None of the patients had evidence of HCC at enrolment based on imaging (ultrasound [US] and, where needed, computed tomography [CT] and/or magnetic resonance imaging [MRI]). Demographic data were retrieved from the patient's files. Seventy percent of the patients had

chronic hepatitis C virus (HCV) infection. Other causes of cirrhosis included chronic hepatitis B virus (HBV) infection, alcohol abuse and autoimmune diseases (Table 1). After enrolment, all patients had careful screening for HCC based on Doppler-US every 6 months or 3 months according to randomization. A standardized report was completed by each operator, mentioning the presence or absence of focal lesions. If focal lesions were present, the localization, number, echogenicity and diameter of nodules were recorded. After the termination of this trial (with a mean follow-up of 4 years), patients were followed according to EASL guidelines[25] with abdomen US every 6 months with or without alpha foetoprotein (AFP) measurement according to center policy. One patient who developed HCC during the first year and six patients with a follow-up shorter than one year were excluded from further analysis (Figure 2).

3.3.2 DESIGN

A glycomic fingerprint including the GlycoCirrhoTest was obtained on serum samples collected at enrolment in this prospective cohort study[24] and stored. Alpha-Foetoprotein (AFP) levels were measured on the same serum samples. FIB-4 was calculated using available laboratory values from the medical records. Diagnosis of HCC was established according to the 2001 EASL criteria[25]. When imaging was not conclusive, patients had a US guided-biopsy. Patient characteristics and routine laboratory values at inclusion are summarised in Table 1.

3.3.3 GLYCOMIC ANALYSIS

Five microliter of serum were processed according to the in-solution deglycosylation method described by Vanderschaeghe et al.[16]. 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[26]. The result of this analysis is a total serum protein electropherogram (Figure 1), which consists of 13 peaks. Each peak represents a well-identified glycan[17]. 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. The GlycoCirrhoTest is calculated as the logarithmic transformation of the abundance ratio of a bigalacto core-α-1,6-fucosylated bisecting biantennary glycan (NA2FB) to a triantennary glycan (NA3)[1].

3.3.4 ALPHA-FOETOPROTEIN ANALYSIS

All serum samples were diluted in 0.9% NaCl and analysed by Electro-chemiluminescence immunoassay (ECLIA) in a MODULAR E module (ROCHE).

3.3.5 STATISTICAL ANALYSIS

Statistical analysis was performed using IBM©SPSS©Statistics Version 22.0. Based on a two sample t-test, mean serum levels of biomarkers were compared between patients that developed HCC and those who did not. For every marker a cox regression analysis was performed with the development of HCC as outcome variable. For cox regression analysis, an internal validation of the model was performed by applying a bias-corrected and accelerated (BCa) bootstrap (n=1000). Based on the relative change in abundance of the 6 glycans that were different (p ≤0.1) at baseline between the group of patients that developed HCC and those who did not, we designed a new composite score, the GlycoHCCRiskScore, based on multivariable logistic regression. GCT, based on two single glycans as described above, was also calculated. For both the GlycoHCCRiskScore and the GCT a ROC analysis was performed and Youden index was used to select an optimal cut-off. The patients were classified according to these cut-offs, and Cumulative Incidence (One minus cumulative survival) was calculated with the Kaplan-Meier method. Cox regression analysis was used to estimate the hazard ratio for HCC development in the biomarker-positive vs. biomarker-negative patient groups. A multivariable cox regression analysis was performed including GlycoCirrhoTest and AFP . Using the *validate* function of the *rms* package in R (version 3.2.3) cross-validation was applied to the logistic and cox regression models to adjust for the optimism in C-Index estimation. Statistical significance was set at the alpha level = 0.05.

3.4 RESULTS

3.4.1 BASELINE CHARACTERISTICS

After exclusion of patients (n=6) with a follow-up of less than 1 year and patients who developed HCC less than 1 year after enrolment (n=2), 125 patients were included for final analysis (Figure 2). Among these patients, 34 (27.2%) developed HCC during follow-up after a mean interval of 66.67 months (SD 29.30). Baseline characteristics of patients who developed HCC were comparable to those who did not, except for time at risk (Table 1). Time at risk was defined as the duration of follow-up until appearance of HCC, liver transplantation, death or loss to follow-up. Six patients died during follow up. Three deaths were related to HCC. Time at risk was higher in the patients who did not develop

HCC, compared to those who did (7.6 years vs. 5.7 years, p=0.012). However, this is inherent to the study design, as follow-up was stopped as soon as the patients developed HCC.

3.4.2 ASSOCIATION OF GLYCOCIRRHOTEST WITH THE DEVELOPMENT OF HCC

GlycoCirrhoTest is calculated as the logarithmic transformation of the ratio NA2FB to NA3 (Figure 1) and was initially developed as a diagnostic marker for cirrhosis[1]. Baseline GlycoCirrhoTest values were significantly higher (Figure 3) in patients who developed HCC during follow-up compared to patients who did not develop HCC (mean GlycoCirrhoTest value : 0.33 vs 0.16, p<0.001).

A cox regression analysis was performed with baseline GlycoCirrhoTest as a single predictor for the risk for developing HCC. The hazard ratio (HR) of GlycoCirrhoTest of 1 for developing HCC was 10.294 (95%CI: 3.37231.426, p<0.001) . Each more modest increase of GlycoCirrhoTest of 0.2, which is more clinically relevant, showed a hazard ratio of 1.59 (95%CI: 1.275-1.993, p<0.001). A bootstrap analysis confirmed these data (p<0.001). The estimated C-Index of this regression analysis was 0.69, after cross-validation the estimated C-Index was 0.69.

A ROC analysis (Figure 3) showed an area under the curve of 0.71 (p=0.001 – 95% CI 0.59-0.80) for the association with HCC development. Based on the Youden index a cut off of 0.2 was defined, yielding a sensitivity of 79% and a specificity of 62%. After cross-validation the estimated c-index was 0.70.

Table 1: Baseline characteristics of cirrhotic patients developing HCC and patients without HCC

	All Patients	No HCC	HCC	
				p-value (Fisher's Exact Test)
Gender (M/F)	96/29 (76.8/23.2%)	68/23 (74.7/25.3%)	28/6(82.3/17.6%)	0.388
Etiology				0.830
HCV	72	51(56%)	21(61%)	
HBV	27	22(24%)	5(14%)	
Alcohol	8	5(6%)	3(9%)	
Alcohol+HCV	8	8(9%)	0(0%)	
Alcohol+HBV	6	1(1%)	5(15%)	
Other	4	4(4%)	0(0%)	
	Mean (+/- SD)	Mean (+/- SD)	Mean (+/- SD)	p-value (Mann Whitney U test)
Age	53.1 (11.1)	52.6 (11.2)	53.9 (10.2)	0.528
Time at risk (years)	6.76 (3.8)	7.6 (3.9)	5.6 (2.6)	**0.018**
Child Pugh	5.3 (0.7)	5.3 (0.7)	5.3 (0.6)	0.825
Total Bilirubin (mg/dl)	1.1 (0.6)	1.1 (0.6)	1.2 (0.4)	0.087
Albumin (g/l)	38 (5.4)	38 (5.7)	38 (4.9)	0.889
Creatinine (mg/dl)	0.81 (0.14)	0.80 (0.1)	0.85 (0.1)	0.109
AST (U/l)	67 (50)	66 (51.9)	67 (46.6)	0.340
ALT (U/l)	75 (69)	74 (68.3)	74 (75.3)	0.872
GGT (U/l)	149 (147)	144 (147.6)	150 (131.5)	1.000
PAL (U/l)	99 (57)	90 (51.2)	122 (77.9)	0.100
Platelets (/µl)	143689 (55860)	143150 (53445)	152125 (53285)	0.618
INR	1.21 (0.21)	1.20 (0.19)	1.22 (0.22)	0.974

As illustrated in figure 4, in patients with a GlycoCirrhoTest value <0.2 at enrolment, the Kaplan-Meier cumulative incidence of HCC after enrolment was 23% for the complete duration of the study. In patients with a GlycoCirrhoTest value ≥0.2 at enrolment, the cumulative incidence of HCC after
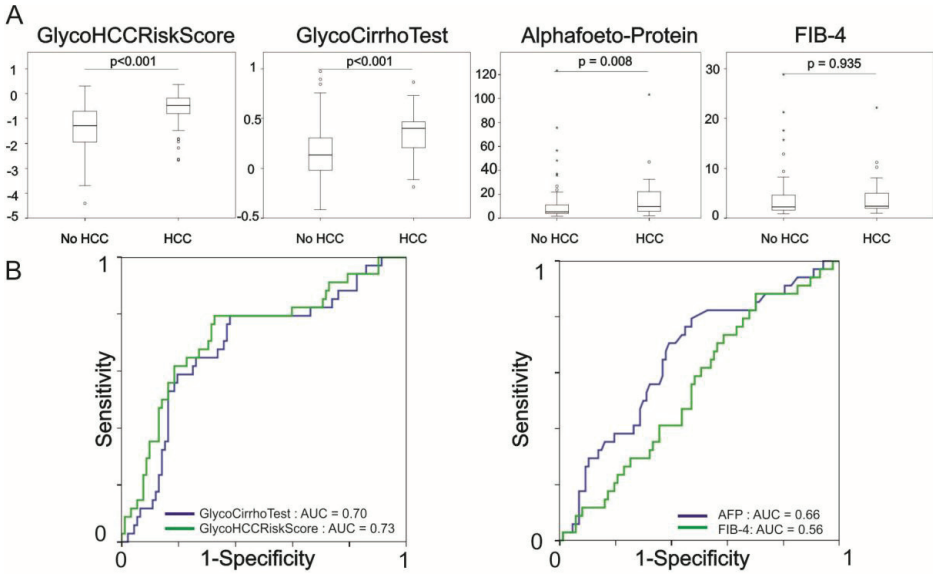
Figure 3. Overview of results *Panel A:* Baseline values of GlycoHCCRiskScore and GlycoCirrhoTest are significantly increased in patients who developed HCC during follow-up (tested by Mann Whitney U test). This was not the case for AFP and FIB-4 values. *Panel B:* ROC analysis showed a AUC for the development of HCC during follow-up of respectively 0.73 (95% CI: 0.063-0.083) and 0.70 (95% CI : 0.59-0;80) for GlycoHCCRiskScore and GlycoCirrhoTest. AFP and FIB-4 failed to show a significant association with HCC occurrence (AUC respectively 0.66 (95% CI 0.59-0.77) and 0.56 (95% CI 0.449-0.65)).

enrolment was 57%. The cumulative incidence of HCC after 5, 7 and 10 years in patients with a GlycoCirrhoTest value above or equal to 0.2 at enrolment was 22.2%, 41.9% and 57.0%. In patients with GlycoCirrhoTest below 0.2 cumulative incidence rates were respectively 3.3, 3.3 and 22.6%. It is clear from visual inspection of these curves that the predictive power for HCC risk of GlycoCirrhoTest stretches is mainly valid for a remarkably long 5-7 years upon serum sampling, upon which the discriminatory power wanes. This is confirmed using ROC analysis for HCC occurrence over different length of monitoring time upon serum sampling. In this cohort, the highest AUC values were reached

for the prediction that a patient would develop HCC within the next 5-6 years (Table 2). After this, the AUC trended downwards again.

Table 2. Time-dependent prognostic value of GlycoCirrhoTest and GlycoHCCRiskScore. Values reflect the area under the curve using ROC analysis with 95% CI (between brackets).

	Number of HCC cases	GlycoHCCRiskScore	GlycoCirrhoTest
HCC within 2 years	3	0.605 (0.274-0.896)	0.438 (0.223-0.829)
HCC within 3 years	6	0.703 (0.401-0.896)	0.643 (0.392-0.869)
HCC within 4 years	10	0.798 (0.633-0.901)	0.742 (0.534-0.902)
HCC within 5 years	14	0.803 (0.566-0.899)	0.766 (0.612-0.889)
HCC within 6 years	17	0.771 (0.610-0.881)	0.742 (0.569-0.871)
HCC within 10 years	34	0.729 (0.602-0.856)	0.691 (0.564-0.791)

Overall hazard ratio for HCC development based on Cox regression analysis was 5.05 (95% CI 2.2-11.7; p<0.001) for the total duration of the study. Taking into account only the first 7 years of follow up, the hazard ratio was 12.1 (95% CI 2.8-51.6; p=0.01).

3.4.3 PROGNOSTIC VALUE OF AFP, FIB-4 AND OTHER BASELINE BIOCHEMICAL VARIABLES

Mean serum AFP levels were slightly increased in the group of patients who developed HCC, as compared to the group of patients who did not (16.09 vs. 10.79 ng/ml; p=0.008). A cox regression analysis showed a significant increase of HCC risk according to baseline AFP value with a hazard ratio equal to 1.018 (95%CI: 1.005-1.031, p=0.008). A bootstrap analysis confirmed these data (p<0.001).

A ROC analysis showed an area under the curve of 0.67 (p=0.005 – 95% CI 0.59-0.77) for the association with HCC occurrence (Figure 3). Again, we defined the cut off using the Youden index. The optimal cut off value for AFP was 5.75, yielding a sensitivity of 76% and a specificity of 55%. In patients with AFP levels below 5.75 at enrolment, the cumulative incidence of HCC was 6.4%. In patients with AFP levels above or equal to 5.75 at enrolment, the cumulative incidence of HCC was 19%.

Overall hazard ratio for HCC development based on Cox regression analysis was 3.21 (95% CI 1.47-7.07; p=0.04) for the total duration of the study. Taking into account only the first 7 years of follow up, the hazard ratio was 4.65 (95% CI 1.59-13.61; p=0.005).

Mean FIB-4 values were comparable between the patients who developed HCC and those who did not (4.01 vs. 3.93, p=0.545). Baseline Child-Pugh score, MELD score, platelets, INR, bilirubin and albumin level were not different between patients who developed HCC and those who did not (Table 1). Using univariable cox regression analysis, none of these markers were significantly related to HCC occurrence.

3.4.4 MULTIVARIABLE COX REGRESSION MODEL INCLUDING GLYCOCIRRHOTEST AND AFP .

Only GlycoCirrhoTest and AFP were significantly associated with HCC occurrence in univariable cox regression analysis. Both were included in a multivariable cox regression analysis. This model confirmed the strong association of GlycoCirrhoTest with HCC development (HR 8.77: 95% CI 2.74-28.08; p<0.001). In contrast, in this multivariable regression model, AFP showed no significant association with HCC development (p=1.165).

3.4.5 TOTAL BASELINE GLYCOMIC FINGERPRINT AND RISK FOR DEVELOPING HCC

The blood serum N-glycan electropherogram yields 13 glycans that have been identified before[26] (Figure 1). The GlycoCirrhoTest is based on only two glycans. Next, we wanted to investigate whether changes in the other 11 glycans generated in the N-glycan analysis could provide additional HCC-predictive information that was not captured by the two glycans of the GlycoCirrhoTest. In the patients who developed HCC during follow up, a significant increase in the relative abundance of NA2FB (p=0.028) and NA3Fb (p=0.023) as well as a significant decrease in the relative abundance of NA3 (p=0.01) and NA4 (p<0.001) were observed. The relative abundance of the remaining glycans did not differ significantly between both groups, although NA3Fbc (p=0.066) and NGA2FB (p=0.056) showed a trend to increase at the α-level of 0.05 (Mann Whitney U-test throughout).
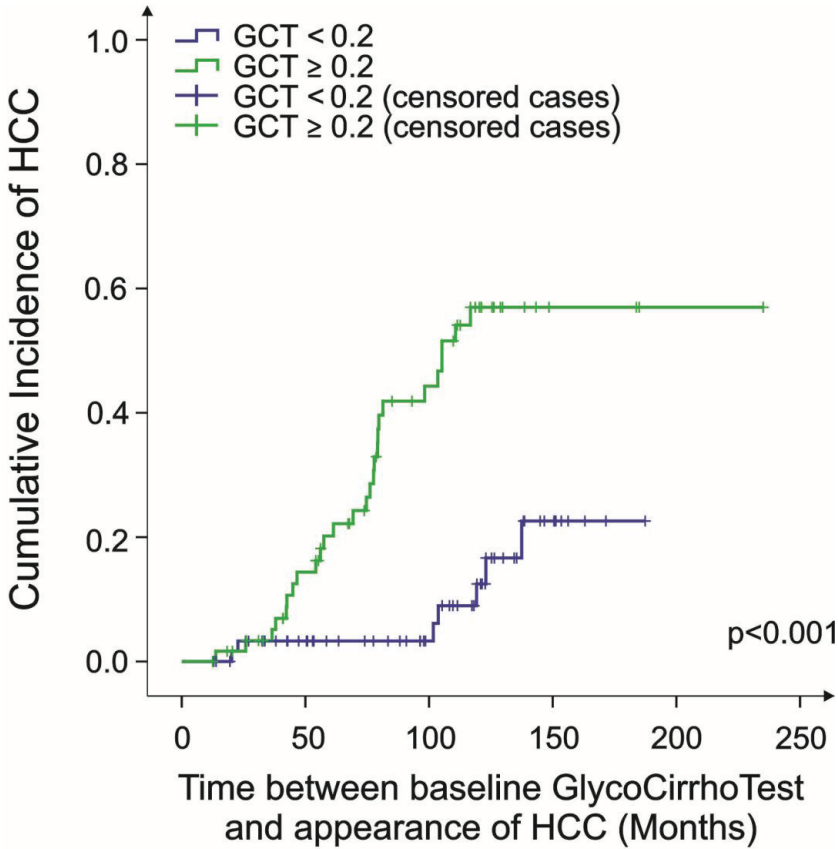
Figure 4: Cumulative incidence curve representing the risk for developing hepatocellular carcinoma according to value of the GlycoCirrhoTest. The cohort was divided according to the GlycoCirrhoTest threshold and monitored for the appearance of hepatocellular carcinoma. The blue line represents patients with a GlycoCirrhoTest value <0.2; the green line represents patients with a GlycoCirrhoTest above or equal to the threshold of 0.2. These patients show an increased risk for HCC development (Hazard ratio = 5.1; 95% CI 2.2-11.7; p<0.001). Censored cases (as indicated by crosses on the cumulative incidence curves) died, underwent liver transplantation or were lost to follow-up.

Based on the relative change in abundance of these 6 glycans that differed at baseline between the group of patients that developed HCC and those who did not (p ≤ 0.1) (Mann Whitney U) we designed a new composite score (GlycoHCCRiskScore) via logistic regression analysis.

GlycoHCCRiskScore=[(NGA2FB*0.137)+(NA2FB*-0.044)+(NA3*-0.216)
+(NA3F*0.158)+(NA3Fbc*0.796)+(NA4*-0.764)].

The algorithm includes the relative increase or decrease of every glycan (beta-value) given by logistic regression analysis. As expected, the mean baseline GlycoHCCRiskScore is significantly increased in cirrhotic patients (-0.69 vs. -1.39, p<0.001) that developed HCC during follow-up (Figure 3).

A cox regression analysis was performed (univariable analysis) to further evaluate the value of baseline GlycoHCCRisk score for association with the risk for HCC development. An increase of GlycoHCCRiskScore with 1 showed a HR of 2.72 (CI 1.69-4.38, p<0.001) for HCC occurrence. An internal bootstrap validation confirmed the statistical significance of this finding (p < 0.001). The estimated C-Index of this cox regression was 0.75, after cross-validation the estimated C-Index was 0.67.

A ROC analysis (Figure 3) was performed for the HCC-prognostic value of GlycoHCCRiskScore and showed an area under the curve of 0.730 (95% CI 0.63-0.83; p<0.001). After cross-validation the estimated C-Index was 0.640. This is not significantly better than the value obtained for GlycoCirrhoTest, indicating that this simple marker comprehensively captures the HCC-hazard relevant information in the total serum N-glycome.

3.5 DISCUSSION

Although many biomarkers have recently been developed for the diagnosis of (early) HCC[3], prognostic markers to stratify patients with compensated cirrhosis with higher and lower risk for HCC are lacking.

We here find that a simple serum glycomics-based biomarker (GlycoCirrhoTest) can be used to assess the risk for the development of HCC in patients with compensated cirrhosis. The role of the liver in the glycosylation of serum proteins is central. GlycoCirrhoTest is based on the ratio of abundance of a bisecting GlcNAc-modified N-glycan and a triantennary glycan on the total mixture of serum proteins. We previously showed that this marker could help identify patients with compensated cirrhosis among patients with chronic liver diseases, with a 79% sensitivity and a 86% specificity[1] in

the discovery cohort at the statistically optimal cutoff value. At the slightly higher cutoff of 0.2 which was found in the present study to be optimal for use of GlycoCirrhoTest as an HCC risk predictor, about 50% of compensated cirrhosis cases surpass this threshold in an aggregate analysis of three independent cohorts from multiple clinical centers (total number of included chronic liver disease patients >600; manuscript in preparation). This corresponds well with the 50% cumulative long-term incidence of HCC observed in the present study for compensated cirrhosis patients with a GlycoCirrhoTest value of higher than 0.2. Although these findings require further validation, this strongly indicates that GlycoCirrhoTest-based monitoring of chronic liver disease patients would reliably and non-invasively detect almost all compensated cirrhosis patients who are at real risk for HCC development. This answers a major medical need in current tools for cirrhosis and HCC monitoring.

The cut-off of 0.2 favours sensitivity above specificity, as the implications of a false positive screening results, which would lead to a supplementary imaging of the liver, is more acceptable than a false negative result. Patients who expressed a GlycoCirrhoTest above 0.2 experienced a significantly increased cumulative HCC incidence of 42% versus 3% after 7 years (p<0.001). Overall hazard ratio for HCC development based on Cox regression analysis was 5.1 (95% CI 2.2-11.7; p<0.001) for patients who had a baseline GlycoCirrhoTest higher than 0.2, and at this same cutoff the hazard ratio for HCC development within 7 years was 12.1 (95% CI 2.8-51.6; p=0.01). In contrast, AFP showed a hazard ratio of 4.65 (95% CI 1.588-13.607; p=0.005). In a multivariable cox regression model including GlycoCirrhoTest and AFP , only GlycoCirrhoTest showed a significant association with the occurrence of HCC. Similarly, the prognostic value of the whole glycomic fingerprint including all 13 glycans, as expressed in the GlycoHCCRiskScore was not superior to the value of the GlycoCirrhoTest. Of note, all of the glycans that had higher abundance in the patients with increased HCC risk were modified with either a fucose, a bisecting GlcNAc or both, whereas the glycans with decreased abundance are the unmodified precursors. This overall glycome change is most simply and robustly captured in GlycoCirrhoTest (AUC = 0.70, which is very similar to the one of the full complexity GlycoHCCRiskScore, AUC = 0.73).

The underlying pathophysiological rationale of the GlycoCirrhoTest is partially elucidated. The enzyme responsible for the formation of bisecting GlcNAc residues, N-acetylglucosaminyltransferase III (GnT-III) is not expressed in hepatocytes in normal physiological conditions, but is increasingly expressed in rat liver dysplastic and malignant nodules during hepatocarcinogenesis[19,20]. Furthermore, GnT-III activity is gradually increased in sera and nodular liver tissue of cirrhotic patients without and with HCC[22,21]. Importantly, we previously showed that GlycoCirrhoTest is specifically increased in cirrhotic patients, but not in patients with earlier stages of liver fibrosis[1],

which supports the hypothesis that GlycoCirrhoTest increase is related to upregulation of GnT-III in regenerative nodules, which are the histological hallmark of liver cirrhosis and are not present in earlier stages of liver fibrosis. It is conceivable that with more hepatocytes actively dividing in such nodules, the risk for propagation of oncogenic mutations increases and hence the risk for HCC rises. Therefore a true marker for such nodular regeneration in liver cirrhosis should also be a good risk marker for HCC, as validated here for GlycoCirrhoTest[1].

Alternative biomarkers for HCC risk have been proposed. FIB-4, a composite biomarker based on AST, ALT, platelet count and age, has been suggested as a prognostic marker for HCC development among chronic hepatitis C infected patients; however, this study included a majority of non-cirrhotic patients, and was not designed to assess risk within a cirrhotic cohort[27]. In contrast, our cohort included only patients with compensated cirrhosis. Within this cirrhotic population, the HCC prognostic power of FIB-4 could not be confirmed. Risk models for the prediction of HCC risk have been developed. Abu-amara et al.[28] recently perfomed an external validation of 5 risk models for HCC development and showed a good prognostic performance, especially for the identification of low risk patients. However, these models have only been developed for chronic hepatitis B patients. It is well known that hepatitis B is an oncogenic virus with appearance of HCC before the cirrhotic stage has been reached in a larger fraction of patients[29]. Hung et al.[30] developed a risk scoring system in an Asian population based on routine clinical parameters, that detects more incident HCC patients as compared to current guidelines, within 10 years. Again, 62% of patients included in this cohort were HBsAg positive. Our cohort covers primarily patients with chronic hepatitis C and alcohol related liver disease, which reflects the European or North American epidemiology. The value of GlycoCirrhoTest in predicting HCC risk in HBV-infected patient populations remains to be determined in future studies.

Current EASL and AASLD guidelines[1011] recommend biannual screening of cirrhotic patients with ultrasonography for the appearance of HCC, which is cost-effective as it results in the detection of more cases of HCC in a stage that can be effectively treated using existing interventions. However, such screening requires the prior diagnosis of cirrhosis, and this is often not the case for patients with early-stage, compensated cirrhosis. Inclusion of GlycoCirrhoTest in the monitoring scheme for chronic liver disease patients can fill this gap, which should ultimately lead to the detection of more HCC cases at a curable stage of the disease than is presently achievable.

Furthermore, using the 0.2 cut-off, GlycoCirrhoTest offers a high 7-year negative predictive value for HCC development of 97%, allowing for the selection of low risk patients. We might imagine that these patients could be offered a less stringent follow-up which is not only more convenient for the

patients but might prove a cost-saving strategy. These observations demand a prospective validation with GlycoCirrhoTest measurement as part of the monitoring strategy for chronic liver disease patients, preferably in current standard clinical practice. The immediate utility of GlycoCirrhoTest may also be to use it as an inclusion criterion for cirrhotic patients in HCC-preventing clinical trials. With a 12 fold increase of 7 year HCC incidence in the GlycoCirrhoTest positive group, the required number of patients for clinical trials could be very significantly reduced, saving cost and time in organising such trials.

Acknowledgements

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IV. The potential of glycomics-based biomarkers to assess the quality of the donor liver graft before liver transplantation using perfusate glycomic analysis

Pretransplant glycomic analysis of perfusate is predictive of primary non function after liver transplantation

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4.1 ABSTRACT

Introduction : Primary non function (PNF) is a rare but major complication after liver transplantation requiring urgent retransplantation. It is associated with the use of extended-criteria donors. The donor risk index is a clinical score that can guide the estimation of graft quality but lacks the power to predict PNF risk in individual patients. Perfusate analysis is an attractive tool for assessment of donor liver function before implantation. Glycomic assessment of serum has proven useful in the diagnosis of liver disease. Here, we performed a comprehensive analysis of perfusate in relation to the appearance of PNF.

Methods: In this prospective monocentric study 66 consecutive liver transplantations between October 2011 and July 2013 were included. Perfusate samples were collected after flushing of the hepatic veins before implantation of the liver graft. All donor grafts were transported using cold static storage. Based on an optimized DNA sequencer technology we performed glycomic analysis of these perfusate samples and searched for glycomic alterations in PNF patients.

Results: The relative abundance of one single glycan, an agalacto core-alpha-1,6-fucosylated biantennary glycan (NGA2F) was significantly increased in the perfusate of the 3 patients that developed PNF after liver transplantation. It could identify PNF patients with 100% accuracy. This glycomarker, called GlycoPNFTest, was the only predictor of PNF in a multivariate analysis (p<0.0001).

Conclusion: In this cohort, patients who developed PNF after liver transplantation showed a specific glycomic signature in perfusate (before liver transplantation) that could distinguish them from non-PNF patients with 100% accuracy. This approach could guide the removal of donor grafts at risk for PNF from the donor pool, especially when the use of high-risk organs is considered.

4.2 INTRODUCTION

Liver transplantation is the treatment of choice for end-stage liver disease, acute liver failure and selected patients with hepatocellular carcinoma[1] with excellent long term results[2]. A major challenge in the expansion of organ transplantation is shortage of donor organs, which is reflected in a unacceptably high waiting list mortality[3]. This bottleneck has led to the use of expanded criteria donors (ECD) donors in order to increase the donor pool[4–6]. Marginal livers or 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 donor[9] 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 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 a constellation of findings that may include hyperbilirubinemia, coagulopathy, encephalopathy or ascites[15]. It occurs in up to 1 in 4 liver transplantations[15]. 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 to predict the appearance of PNF or EAD after liver transplantation are not available. Here, we performed a comprehensive analysis of N-glycans in perfusate in relation to the appearance of PNF and EAD after LT.

Indeed, during the last ten years it has become clear that N-glycan analysis of serum, also called glycomics, is useful for the diagnosis of various liver diseases[17]. After the discovery that a glycomic based serum biomarker can reliably distinguish different stages of liver fibrosis from liver cirrhosis[18,19], glycomics-based biomarkers for the diagnosis of hepatocellular carcinoma (HCC)[20,21] and a biomarker to distinguish simple liver steatosis from non-alcoholic steatohepatitis[22,23] based upon N-glycan analysis have been developed.

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. The second aim was to search for distinct N-glycan profiles in patient that developed PNF, EAD and patients with a good liver graft function, in order to develop biomarkers to monitor organ quality and predict PNF.

The results of the present study provide a strong proof of concept that the analysis of N-glycans in preservation fluid can predict the appearance of PNF after liver transplantation.

4.3 MATERIALS AND METHODS

4.3.1 STUDY POPULATION

This monocentric study was performed at the Liver Transplant Unit of Ghent University Hospital (Belgium) between October 2011 and July 2013. In this period, 85 orthotopic liver transplantations were performed in 74 adult patients at Ghent University Hospital. In this cohort of patients, perfusate from 66 liver transplantations in 64 patients could be sampled prospectively. Recipient and donor characteristics are summarized in table 1.

PNF was defined as the need for urgent retransplantation when a graft did never demonstrate any evidence of initial function following transplantation after exclusion of other causes like hepatic artery thrombosis or acute cellular rejection. It was accompanied by 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].

4.3.2 DESIGN

Every liver transplant patient was eligible as described before. The perfusate samples were collected upon arrival at the recipient hospital during the backtable procedure, after transport of the donor liver. A glycomic analysis of these perfusate samples was performed and the glycomic profile was related to the outcome parameters PNF and EAD.

4.3.3 SAMPLE COLLECTION AND PROCESSING

The perfusate sample was obtained during the back-table procedure. Liver grafts were procured according to local practices and transported to our center in Histidine-trypotophan-ketoglutarate (Custodiol HTK, Franz Köhler Chemie GmbH, Bensheim, Germany) or Wisconsin solution (UW Belzer, Costorsol, Bridge to Life, London, United Kingdom). During the back-table procedure, the left hepatic vein was flushed with a syringe of 20 ml of perfusate in which the graft had been transported under normal hydrostatic pressure. Perfusate samples were collected and immediately stored at -21°C.

Table 1. Patient Characteristics. Patients were divided according to the occurrence of PNF or EAD. Variables between groups were compared using Kruskal-Wallis test. Continous variables are expressed as means followed by standard deviation (SD) between brackets. *Highest INR value betweed postoperative day 2 and day 7. **Highest total bilirubin value between postoperative day 2 and 7

	No PNF/EAD (n =44)	EAD (n=19)	PNF (n=3)	p-value
Recipient Characteristics				
Demographic data				
Age	49.68 (13.64)	52 (15.09)	50 (19.16)	p=0.704
Sex (M/F)	26/18	13/6	2/1	
Biochemical data				
AST at 48 hours (U/L)	594 (446)	2194 (2138)	5488 (6195)	**0.001**
ALT at 48 hours (U/L)	592 (456)	1574 (1461)	1384 (1271)	**0.004**
INR*	1.58 (0.76)	2.17 (1.13)	3.52 (0.96)	**0.001**
Total bilirubin** (mg/dl)	2.91 (3.06)	6.06 (6.55)	11.73 (8.40)	**0.006**
Donor Characteristics				
Age	74.89 (17,69)	47.68 (16.88)	47.67 (18.23)	0.989
Sex (M/W)			2/1	
Length (cm)	172.80 (6.75)	174.32 (7.42)	171 (7.8)	0.310
Weight (cm)	77.08 (11.76)	84.84 (23.41)	60.50 (6.34)	0.093
Perfusate (HTK/UW)	33/10	12/6	2/1	
CIT (minutes)	422.18 (121.68)	389.42 (102.338)	493 (9.90)	0.763
WIT (minutes)	36.73 (12.23)	42.26 (19.36)	40 (11.31)	0.473
AST (U/L)	55.43 (51.91)	63.05 (61.85)	22.50 (3.53)	0.705
ALT (U/L)	42.08 (39.65)	53.53 (65.67)	25.50 (19.09)	0.611
GGT (mg/dl)	69.53 (101.94)	59.26 (44.20)	20.50 (7.79)	0.704
Total Bilirubin (mg/dl)	2.43 (5.77)	2.14 (3.85)	0.72 (0.17)	0.925
DRI	1.67 (0.36)	1.69 (0.41)	1.71 (0.35)	0.897
DCD	0	3	0	

4.3.4 N-GLYCOSYLATION ANALYSIS

All samples were defrosted and analysed in one run. The glycomic analysis was performed in duplo. Perfusate samples were prepared using the 96 well on membrane deglycosylation method, that has been described in detail previously[24]. In summary, N-glycans bound to proteins in 500 µl of preservation solution were denatured using 1 ml of denaturing buffer (10 mM Ammonium Bicarbonate 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, Unit for Medical Biotechnology, Department for Molecular Biomedical Research, VIB—Ghent University, Ghent, Belgium) and labeled with 8-aminopyrene-1,3,6-trisulphonic acid (ATPS - 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. The nature of these peaks has been identified before by structural analysis[18].

4.3.5 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.

4.3.6 ETHICS

The study conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the Ghent University Hospital ethics committee.

4.4 RESULTS

4.4.1 PATIENT POPULATION

N-glycan analysis was performed in perfusate from 66 liver transplantations in 64 patients. Three patients developed a PNF, 19 patients developed EAD and the remaining 44 patients showed a normal liver function after transplantation (No PNF/EAD) patients. In this last group, 2 patients needed an urgent early retransplantation, both due to a hepatic artery thrombosis, and were not considered as PNF. Recipient and donor characteristics are summarized in table 1. Significant differences were observed between the three patients groups regarding recipient ALT and AST values on postoperative day 2, and peak INR and bilirubin levels in the first week after liver transplantation, reflecting the presence of EAD and/or PNF. Interestingly, there were no DCD donors in the PNF group (table 1 and 2).

Table 2. Patient Characteristics. Pairwise comparison of statistically significant different values between patients with normal recovery after liver transplantation, EAD and PNF. Variables between groups were compared using Mann Whitney U test.

Variable	Comparison between groups	p value
Recipient AST at 48 hours	No PNF-PNF	0.507
	No PNF-EAD	**0.001**
	PNF-EAD	1
Recipient ALT at 48 hours	No PNF-PNF	0.763
	No PNF-EAD	**0.003**
	PNF-EAD	1
Recipient INR	No PNF-PNF	**0.011**
	No PNF-EAD	**0.019**
	PNF-EAD	0.347
Recipient Total Bilirubin	No PNF-PNF	0.079
	No PNF-EAD	**0.027**
	PNF-EAD	0.982

4.4.2 N-GLYCAN 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 analysis[21] and has been used for urine analysis[22]. Now, this technique was applied on perfusate from liver transplant patients. Using the standard protocol, we obtained electropherograms with 13 peaks, identically as what has been observed in serum and urine.
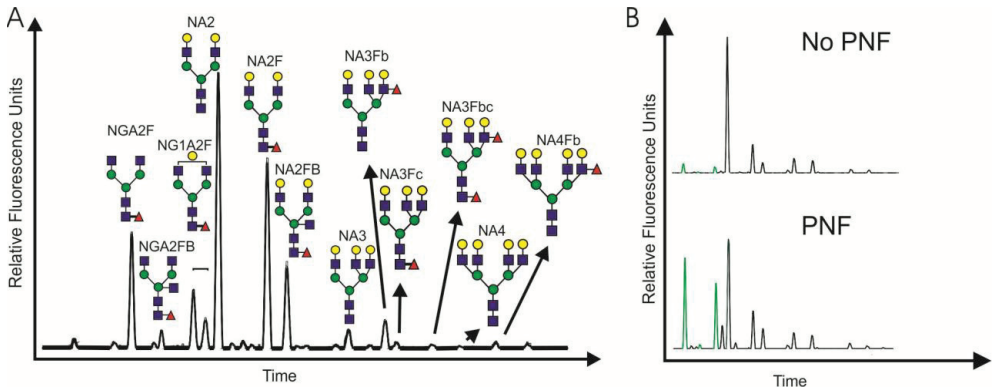
Figure 1. The structures of the N-glycan peaks in a human perfusate sample.

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 monogalacto, 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.

4.4.2.1 Development of a glycomic based biomarker

A desialylated serum protein N-glycan profile was obtained for the 66 samples in the study (Figure 1). We quantified 13 peaks that were well detectable in all samples and normalized their abundance to the total peak height intensity per patient. Peak 1, 2, and 3 showed discriminative changes according

to development of PNF and EAD (Figure 2). However, as can be appreciated from figure 2, PNF patients have a discriminative glycomic signature compared to non-PNF patients, whereas 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 (table 3) 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 glycan (NGA2F)(Figure 2) compared to the EAD and non-PNF/EAD patients (p<0.0001) with an accuracy of 100%.

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). This marker was called the GlycoPNFTest.

4.4.2.2 AST and ALT in perfusate

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.

4.4.2.3 Prognostic markers for PNF

Next we analyzed whether other donor- or recipient parameters were predictive for the occurrence of PNF using an univariable analysis (Mann Whitney U test). None of the clinical donor parameters were predictive for the development of PNF after liver transplantation, nor was AST and ALT analysis in perfusate (Table 3). In a multivariate logistic regression model including NGA2F, DRI, AST and ALT in perfusate, only the glycomarker NGA2F was an independent predictor of PNF (p<0.0001).
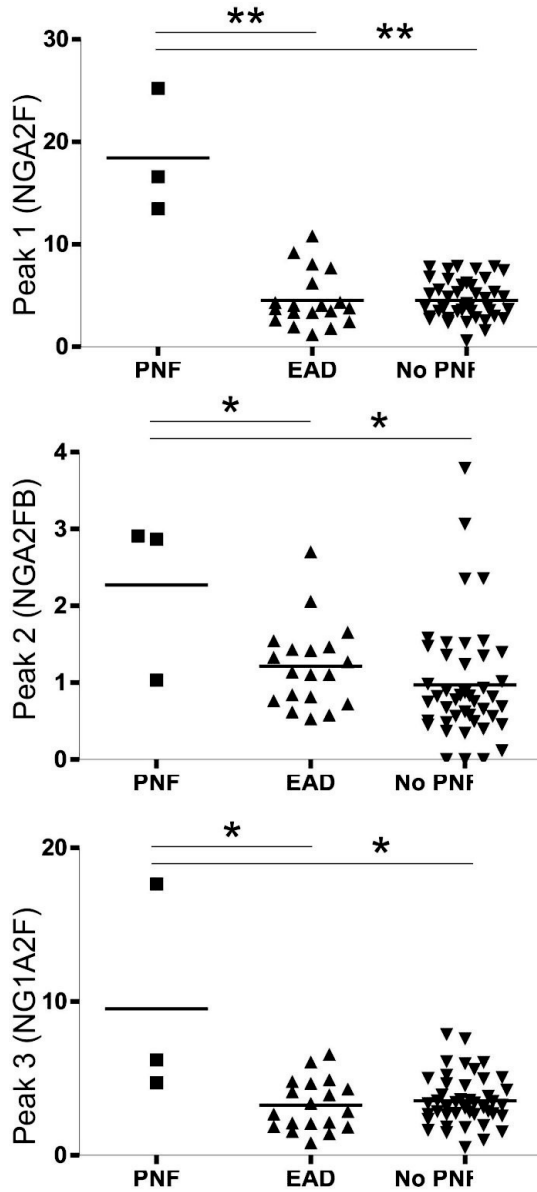
Figure 2. Overview of the relative abundance of relevant glycans in patients that developed PNF afterward. Peak 1 (NGA2F) shows 100% accuracy for prediction of PNF. P values were calculated using Mann Whitney U- test. * p< 0.05, ** p<0.001

Table 3. Prognostic markers for the development of PNF. A. Univariable analysis (Mann Withney U test) was performed in order to look for prognostic markers for PNF development. B. Multivariate logistic regression model. *Highest INR value betweed postoperative day 2 and day 7. **Highest total bilirubin value between postoperative day 2 and 7

A.

Univariable analysis	p-value
Donor Characteristics	
Age	0.954
Sex (M/W)	0.772
Length (cm)	0.269
Weight (cm)	0.175
Perfusate (HTK/UW)	0.858
CIT (minutes)	0.794
WIT (minutes)	0.518
AST (U/L)	0.641
ALT (U/L)	0.908
GGT (mg/dl)	0.871
Total Bilirubin (mg/dl)	0.734
NHBD	0.908
DRI	0.772
Perfusate analysis	
AST	0.653
ALT	0.589
Peak 1	**<0.0001**
Peak 2	**0.042**
Peak 3	**0.010**
Peak 4	0.817

B.

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

4.5 DISCUSSION

We propose a new biomarker, the GlycoPNFTest, based on glycomic analysis of perfusate that might be 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 assessment. Secondly, this glycomic marker could prevent futile transplantations. Indeed, in this cohort, not any clinical parameter was able to predict PNF, in contrast to the glycomarker that could with 100 % sensitivity and specificity differentiate between PNF and non-PNF patients. To our knowledge, not a single biomarker has proven the same utility today. We are however aware that PNF was only present in only 3 patients (what does reflect the low prevalence of PNF). A validation study is ongoing in order to study these findings in a larger amount of patients in a multiple transplant centers. Interestingly, this biomarker has a high negative predictive value for the occurrence of PNF, as the non-PNF patients are clustered within a small range, thus raising arguments to be able to transplant a patient safely without a major risk for PNF development.

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 PNF[25]. 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 clinically acceptable livers[26]. In our cohort, AST and ALT levels showed a wide range among all patients, without distinct correlation with EAD or PNF. Xanthine, an adenine 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 study[27]. In the same patients, adenine nucleotides were decreased in liver tissue after transplantation, reflecting irreversible injury and necrosis of the liver and the retardation of the recovery of cellular adenoside triphosphate (ATP) after reperfusion[28,29]. The group of Starzl showed 20 years ago that hyaluronic acid (HA) levels in effluent higher than 400 µg/L 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 µg/L cut-off showed a normal clinical outcome[30]. Similar findings were retained in a validation study[31], again positioning the test as a marker for identifying safe livers.

The proposed glycomarker for PNF prediction managed to identify the 3 patients with PNF, 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 IgG[19], 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[32,33]. IgG undergalactosylation is a marker of necroinflammation also found in patients with rheumatoid arthritis[34] and increasing levels of liver fibrosis[19]. 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 undergalactosylated glycan and is believed to represent the inflammatory disturbances in the donor liver that will fail after transplantation.

The accuracy for the prediction of PNF was extremely high (100%). On the other hand we found no clear relationship between the perfusate glycomic profile and the occurrence of EAD. We speculate that this might 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. This information will of course not be captured in the pretransplant glycomic profile. 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. However, in one patient 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.

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 perfusion[25], and the role of this glycomarker needs to be explored in machine perfusion.

A drawback in the application of this technique is the turnover time that requires 48 hours. However, sample preparation for serum samples has been simplified and can be used on cheap and widely available high-throughput microfluidics CE platforms including the MCE-202 MultiNA, 2100 Bioanalyzer and eGene system[35]. Validation of the technique for perfusate is ongoing in order to reduce the turnover time to a clinically acceptable time frame.

In conclusion, the study provides a strong proof-of-concept that the glycomic analysis of perfusate predicts with 100% accuracy the occurrence of PNF. A validation of these data in a multicenter clinical trial is ongoing.

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V. The potential of glycomics-based biomarkers to assess the outcome after liver transplantation in the early transplant period using serum glycomic analysis

A glycomic serum marker is predictive for graft loss and patient death during the first year after liver transplantation

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5.1 ABSTRACT

Background : Graft failure after liver transplantation (LT) remains a challenge for transplant professionals and sometimes requires retransplantation. Pretransplant estimation of graft function using scores like donor risk index has limited use in individual patients. Diagnosis of early allograft dysfunction after liver transplantation by clinical criteria can predict graft survival. However, biomarkers that reliably identify patients at risk for graft failure after LT are lacking. Analysis of N-glycans in serum (glycomics) has shown to reflect the underlying liver function in liver disease but has never been assessed after liver transplantation.

Aims : The aim of this study was to assess the potential of serum glycomics as predictive markers for graft loss after liver transplantation.

Methods: Serum glycomic profiles were analysed before and after liver transplantation in 127 liver transplant recipients using an optimized glycomic technology on a DNA sequencer. The major outcome parameters (graft and patient survival) were related to the observed glycomic alterations.

Results : The assessment of 2 serum glycans, NG1A2F (an agalacto, core-alpha-1,6-fucosylated biantennary glycan structure) and NA3 (a triantennary glycan), combined as *log(NG1A2F/NA3)* on day 7 after liver transplantation was strongly associated with graft loss (hazard ratio = 7.222; p<0.001; 95% CI 2.352-22.182) and patient death (hazard ratio = 3.885; p=0.30; 95% CI 1.127-13.276) during the first year after liver transplantation (cox regression analysis). In multivariate cox regression model including early allograft dysfunction (according to Olthoff) and Donor Risk Index, this biomarker, called GlycoTransplantTest, was the only independent predictor of graft survival (p=0.003).

Conclusion : Assessment of GlycoTransplantTest, a glycomic serum marker, on day 7 post liver transplantation is a strong predictor of graft and patient survival during the first year after liver transplantation.

5.2 INTRODUCTION

Since the first successful orthotopic liver transplantation (LT) by Starzl in 1963[1], LT has become the treatment of choice for end stage liver disease and selected patients with hepatocellular carcinoma (HCC)[2]. 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[3]. Graft loss occurs in 7 to 10% of adults[4] and requires

retransplantation in these patients[5]. Retransplantation 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). The donor graft quality is increasingly recognized as a major driver of post-transplant outcome. Moreover, the shortage of donor organs has lead 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[6].

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)[7] 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[8]. A European donor risk index was developed using the Eurotransplant (ET) database resulting in the ET-DRI[9]. 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[10][11][12].

Posttransplant 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[13][14]. The most widely accepted definition for EAD has been validated by Olthoff[13] and is based on the presence of one or more of the following postoperative laboratory analyses: bilirubin >or=10mg/dL on day 7, international normalized ratio >or=1.6 on day 7, and alanine or aspartate aminotransferases >2000 IU/L within the first 7 days after LT. Other scores are based on single measurement of (peak) AST or ALT values[15], bilirubin[12], lactate[16], factor V[17] and platelet counts[18] but do not increase the diagnostic power of this definition. Functional tests include the indocyanine green (ICG)[19,20] – plasma disappearance rate and the liver maximal function capacity (LiMax)[21]. 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[22]. Glycomic analysis of whole serum can be easily performed using a glycan analytical method that uses standard DNA-sequencing equipment[23][24]. Based on this paradigm, we developed several biomarkers based on specific glycoalterations for the diagnosis of liver fibrosis[25] and cirrhosis[24], HCC[26,27] and NASH[28,29].

In this manuscript we studied glycomic alterations in the early post-transplant period and defined a glycomic signature on day 7 after LT , called GlycoTransplantTest, that predicts organ and patient survival during the first year after liver transplantation.

5.3 MATERIALS AND METHODS

5.3.1 PATIENTS

The patients in this monocentric prospective cohort underwent liver transplantation in the Liver Transplant Unit of Ghent University Hospital (Belgium) between 1 December 2012 and 31 December 2014.

5.3.2 DESIGN

From every patient one serum sample was collected in the 24 hour timeframe before liver transplantation. After liver transplantation daily serum samples were collected during 14 days, and after 1,3,6 and 12 months after liver transplantation. After centrifugation, serum samples were frozen to minus 21° Celsius. 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 outcome.

5.3.3 GLYCOMIC ANALYSIS

Five microliter of serum were processed according to the in-solution deglycosylation method described by Vanderschaeghe et al.[25]. 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[23]. 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[30]. 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.

5.3.4 STATISTICS

Statistical analysis was performed using IBM©SPSS©Statistics Version 22.0. Based on Mann Whitney U test, clinical characterstics were compared between patients that developed graft loss and those who did not. ROC analysis was performed and the Youden index was used to select an optimal cut-off. Logistic regression was performed to calculate the relation to the different outcome variables at every defined time-point (2 weeks, 1 month, 3 months and 12 months after LT). The patients were classified according to these cut-offs, and cumulative survival was calculated with the Kaplan-Meier method. Cox regression analysis was used to estimate the hazard ratio for graft loss and death for potential prognostic biomarkers. Finally a multivariable cox regression analysis was performed. Statistical significance was set at the alpha level = 0.05.

5.3.5 ETHICS

The study conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the Ghent University Hospital ethics committee.

5.4 RESULTS

5.4.1 PATIENT CHARACTERISTICS

In this study, 127 liver transplant patients were eligible. However, finally 113 patients were included in the final analysis as the serum sample on postoperative day 7 that is crucial for the measurement of the newly developed GlycoTransplantTest was not available (n=10) or because the patient underwent retransplantation before day 7 due to primary non function (n=4). Seven liver transplants were retransplantations, 1 one was a second retransplantation.

Baseline characteristics are summarized in table 1. In this cohort, 3 patients died within 1 month after LT and 11 patients died within the first post transplant year. Eight deaths were directly liver-related (ischemic cholangiopathy (n=5), small for size syndrome (n=1), HCV recurrence (n=2), 2 were

transplant-related (sepsis from abdominal source, n=1; sepsis from respiratory source, n=1), one patient death was not transplant related (rapidly progressive pharyngeal carcinoma).

Table 1. Baseline characteristics of the liver transplant patients (n=113).

		Number	Percentage
Mean Age	50.65 (SD 13.52)		
Mean MELD			
Sex	Male	61	54
	Female	52	46
Indication	Alcoholic cirrhosis	35	31.0
	PSC	13	11.5
	HCV	12	10.6
	ALF	11	9.7
	Polycystic Disease	8	7.1
	ITBL	7	6.2
	HBV	5	4.4
	NASH	4	3.5
	AIH	4	3.5
	Hyperoxaluria	3	2.7
	HCC	3	2.7
	Other*	8	7.1
Retransplantation (1)		7	6.2
Retransplantation (2)		1	0.9

*other : adenomatosis (2), primary non function (2), Budd Chiari syndrome (1), Familial amyloid polyneuropathy (1), chronic allograft rejection (1), hemangioendothelioma (1)

Graft loss was observed in 4 patients within 2 weeks after LT, in 9 more within the first month, in 4 more within the first 3 months, and in 5 more patients within the first year after LT. Graft loss was related to ischemic cholangiopathy (n=9), sepsis (n=7), hepatic artery thrombosis (n=2), HCV recurrence (n=2) and severe EAD (n=2).

A

Relative Fluorescence Units

Time

NGA2F
NG1A2F
NA2
NA2F
NA3Fb
NA3Fbc
NA4Fb
NGA2FB
NA2FB
NA3
NA3Fc
NA4

B

Pretransplant - Cirrhosis

NA2FB   NA3

Posttransplant - No Cirrhosis

Relative Fluorescence Units

Time

C

GlycoTransplant Test - High

NG1A2F   NA3

GlycoTransplant Test - Low

Time

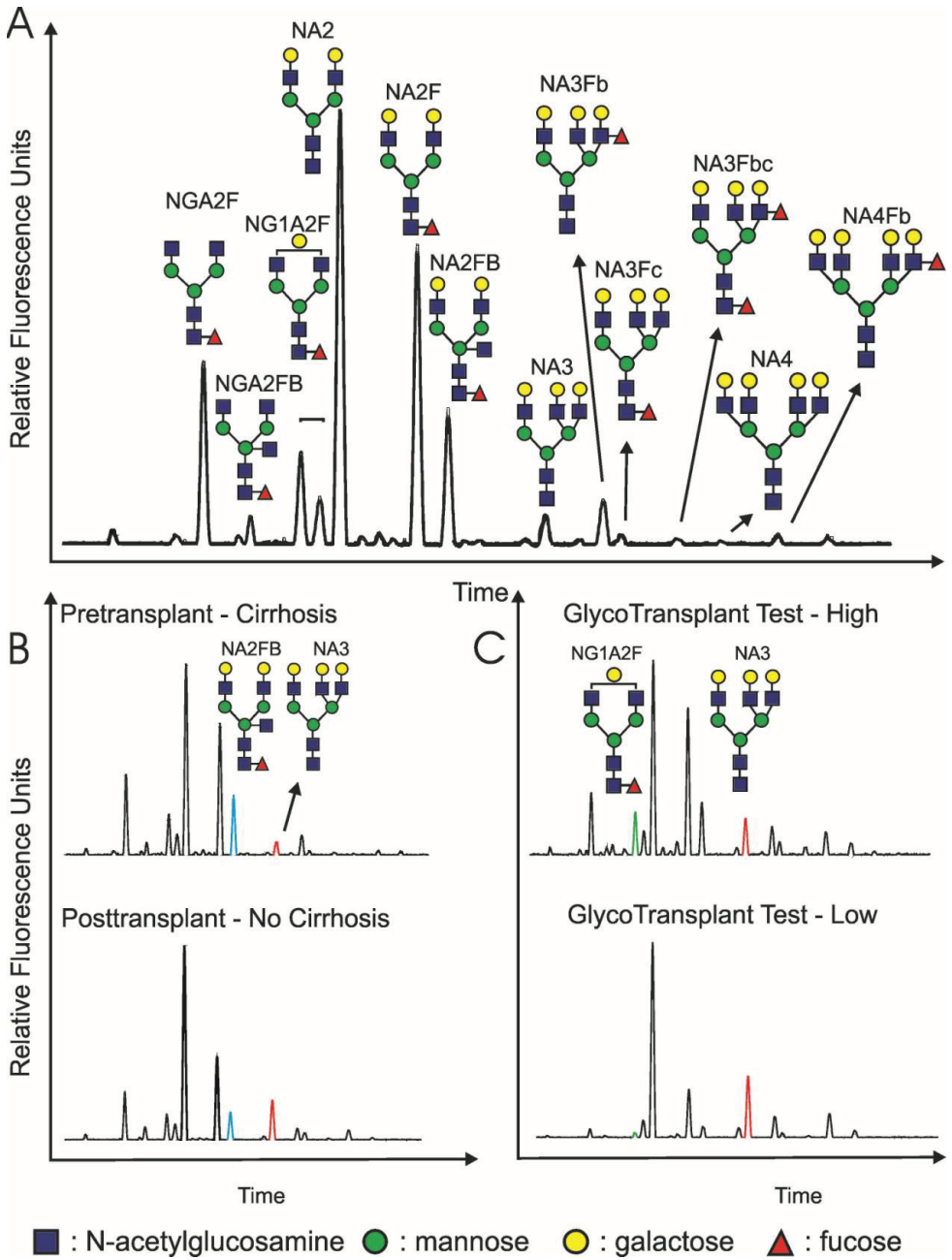■ : N-acetylglucosamine   ● : mannose   ○ : galactose   ▲ : fucose

Figure 1 : The glycomic analysis and GlycoTransplantTest

Panel A: 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 bisecting biantennary (NGA2FB), peak 3 and peak 4 are single monogalacto, 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 9 is the core-alpha-1,6-fucosylated triantennary glycan NA3Fc, 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.

Panel B: Glycomic profile before and 24 hours after liver transplantation in the same patient with liver end-stage cirrhosis. Note the rapid changes and disappearance of the cirrhotic glycomic signature, formerly published as GlycoCirrhoTest[24]. The GlycoCirrhoTest driving rise of NA2FB disappears 24 hours after liver transplantation.

Panel C: The newly developed **GlycoTransplantTest** is based on the increased abundance of peak 3, an agalacto, core-alpha-1,6-fucosylated biantennary glycan (NG1A2F) and the decreased abundance of peak 8, a triantennary glycan (NA3). Upper panel shows a patient with a high score on the GlycoTransplantTest with a bad post-transplant outcome; the lower panel shows a low score in a patient with a good post-transplant outcome.

Mean DRI in patients who experienced graft loss or death was 1.804 (SD 0.382) compared to 1.679 (SD 0.386) in patients without graft loss or death (p=0.189).

5.4.2 DEVELOPMENT OF THE GLYCOTRANSPLANTTEST

Analysis of the whole serum glycome after liver transplantation using DSA-FACE yields a desialylated glycomic profile consisting of 13 peaks (Figure 1). Every peak represents an N-glycan and the value of the peak height is normalized on the total value of the peak heights (sum of all 13 peaks), thus enabling comparison of relative peak heights between different serum samples. The specific structure of these 13 N-glycans has been identified before (Figure 1)[23,24].

We searched for a specific glycomic signature in patients with increased risk of graft loss or death during the first year after liver transplantation, based on a serum glycomic assessment during the first week after liver transplantation.

A logistic regression was performed (univariate analysis) for every glycan in relation to graft survival at 2 weeks, 1 month, 3 months, and 1 year and in relation to patient survival at 1 month and 1 year (supplementary data). Looking for the glycans showing the most complete coverage of deleterious outcome for graft of patient, the highest prognostic value was observed on day 7 after LT. In patients with adverse post-transplant outcome we observed an increase of the undergalactosylated glycans NGA2F, NGA2FB and the NG1A2F isomers, an increase of NA2F and NA2FB and a mild increase of NA2 and the tetraantennary glycan NA4FB. On the other had in these patients, a decrease was observed of the triantennary glycans NA3, NA3FB, NA3FC, NA3FBC, a mild decrease of the tetraantennary glycan NA4. Only 7 glycans showed significant changes according to patient outcome (supplementary file 1).

After further analysis, the appearance of a graft loss or patient death could be captured by only 2 glycans, an increase of peak 3, an agalacto, core-alpha-1,6-fucosylated biantennary glycan (NG1A2F) and a decrease of peak 8, a triantennary glycan (NA3). This signature is summarized in only one variable (log(NG1A2F/NA3)), called the GlycoTransplantTest.

5.4.3 PROGNOSTIC PERFORMANCE OF THE GLYCOTRANSPLANTTEST

Graft survival

There is a strong correlation between the GlycoTransplantTest, based on the measurement of 2 single glycans on day 7 after liver transplantation, and graft loss (Figure 2). Based on ROC curve analysis the area under the curve (AUC) for graft loss 2 weeks after LT was 0.824 (p=0.028; 95%CI 0.597-1.000), for graft loss 1 month after LT it was 0.810 (p<0.0001; 95%CI 0.686-0.934), after 3 month 0.816 (p<0.0001; 95%CI 0.712-0.920) and after 1 year 0.784 (p<0.0001; 95%CI 0.684-0.884). The odds ratio's for graft loss for an increase of GlycoTransplantTest with 1 (univariate analysis) were

respectively 9.165 (p=0.012; 95%CI 01.636-51.343), 7.865 (p<0.0001; 95%CI 2.521-24.539), 8.118 (p<0.0001; 95%CI 2.784-23.675) and 5.844 (p<0.0001; 95%CI 2.398-14.243).

Considering the strongest prognostic performance for prediction of graft loss 3 months after liver transplantation, we defined a cut-off for the GlycoTransplantTest. From the ROC analysis the Youden –index pointed at an optimal cut-off of -0.81 showing a sensitivity of 82.4% and a specificity of 70.5%.

The Kaplan-Meier curve shows a significant discrimination regarding graft survival (Log Rank p<0.0001) when using this cut-off for the GlycoTransplantTest (Figure 3), with patients above the cut-off of –0.81 showing a worse graft survival.

Using cox regression analysis (univariate), the hazard ratio for graft loss according to the GlycoTransplantTest cut off was 7.222 (p<0.001; 95% CI 2.352-22.182).

Patient survival

Based on ROC analysis, the GlycoTransplantTest was not related to patient death after 1 month (AUC 0.817, p=0.062) but was related to patient death after one year (AUC 0.702, p=0.028; 95%CI 0.566-0.838). The odds ratio for patient survival after 1 month and 1 year for an increase of GlycoTransplantTest with 1 (univariate analysis) were not significant.

Based on the Youden–index the optimal cut-off was -0.74 showing a sensitivity of 63.3% and a specificity of 70.1%. Using the same cut-off of –0.81 as for graft survival, sensitivity was 63.3% and specificity 65.3%.

Using cox regression analysis (univariate), the hazard ratio for patient death according to the optimal GlycoTransplantTest cut off of –0.81 was 3.885 (p=0.030; 95% CI 1.127-13.276). The discriminative value is again illustrated by the Kaplan-Meier curve (Log Rank test: p= 0.020) (Figure 3).

5.4.4 PROGNOSTIC PERFORMANCE OF AST AND ALT

In order to challenge the prognostic potential of the GlycoTransplantTest, we studied the prognostic potential of the routinely analysed transaminases. AST and ALT were analysed on daily bases during the first week after transplantation. Only during the first 4 days after LT, AST and ALT were significantly related to these outcome variables. In literature peak transaminases during the first three days after LT have been attributed a prognostic power for graft and patient survival. Based on this knowledge, we focused on AST and ALT in the first 3 days after LT[15,31].
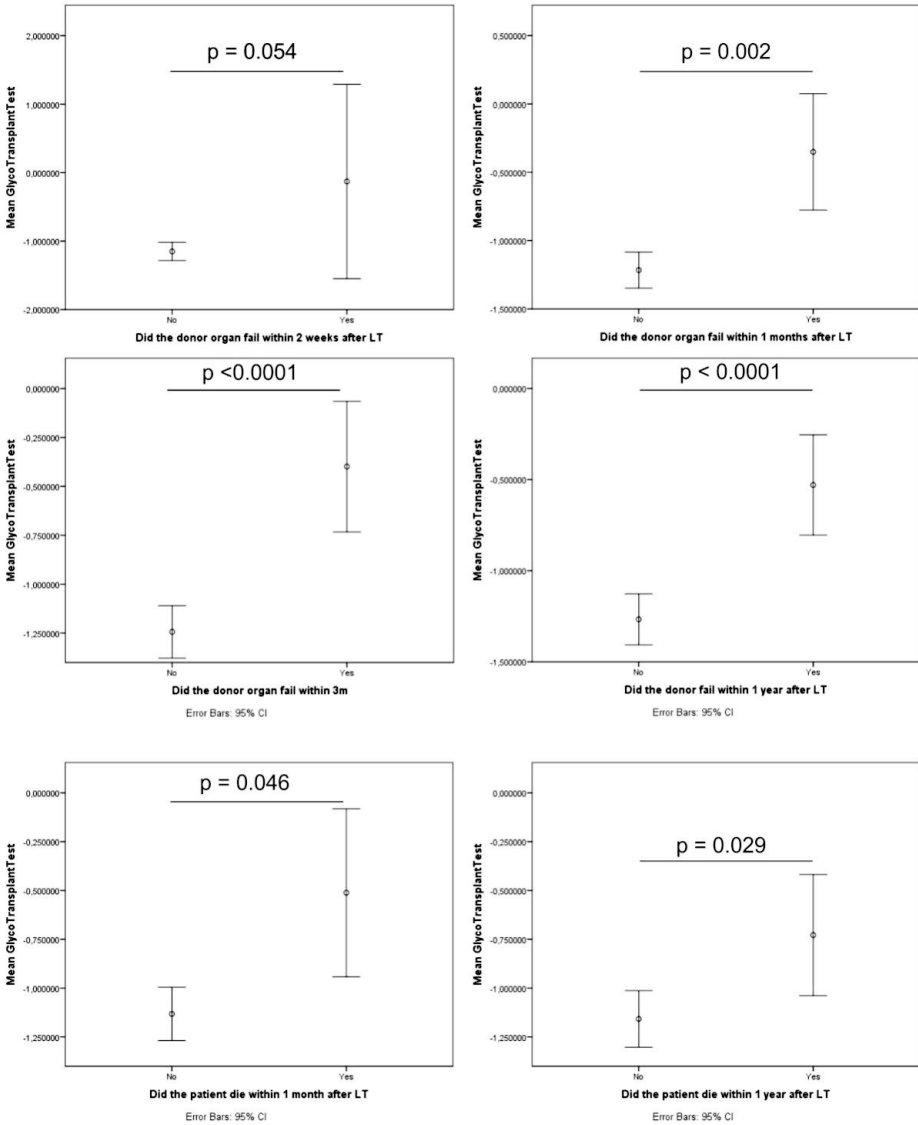
Figure 2. The GlycoTransplantTest is significantly increased in patients with worse outcome. Mean values with 95% confidence intervals are presented. Comparison between groups was performed using Mann Withney U test.
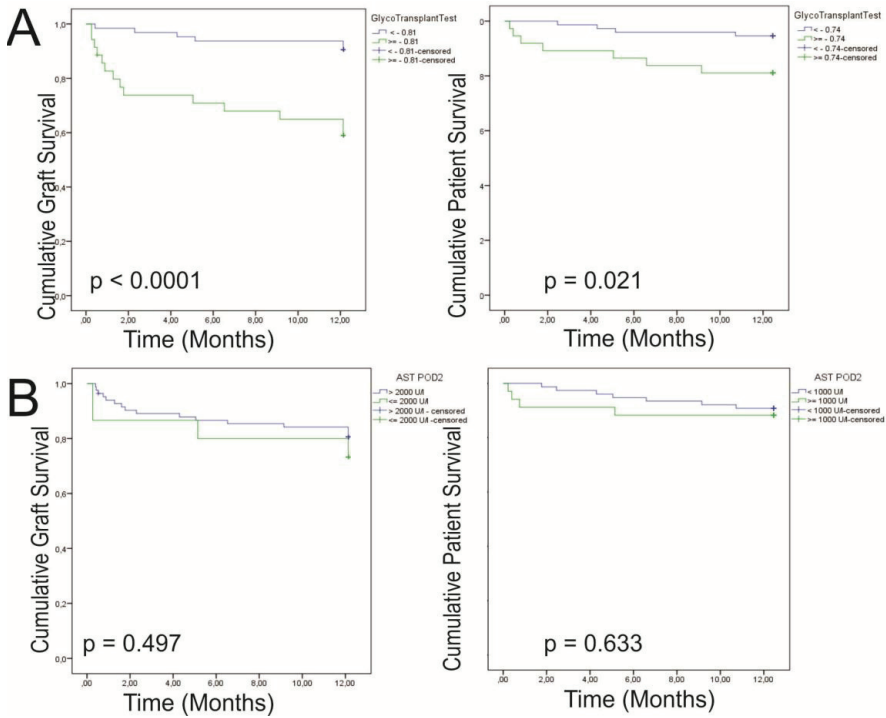
Figure 3. Prognostic performance of the GlycoTransplantTest compared to AST on postoperative day 2. Kaplan Meier curves are shown according to the defined cut-off. GlycoTransplantTest shows a better discriminative value than AST on POD2 between patients with graft loss versus graft survival (left panel) and between patients who die during the first year after LT versus survivors (right panel). P values were calculated using Log rank test.

Graft survival

ROC analysis for AST and ALT on postoperative day (POD) 1 , POD2 and POD 3 for prediction of graft loss after 2 weeks, 1 month, 3 months and 1 year was performed. AUC showed disappointing values between 0.531 and 0.740, without statistical significance.

However, after logistic regression (univariate analysis), AST on POD 2 showed a significant association with graft loss at 1 month (OR 1.194, p=0.006; 95% CI 1.051-1.356), 3 months (OR 1.138, p=0.034; 95% CI 1.010-1.282) and 12 months (OR 1.129, p=0.041; 95% CI 1.005-1.269). AST on POD 3 was significantly associated with loss of transplant organ at 1 month (OR 1.311, p=0.029; 95% CI 1.028-

1.672). ALT on POD2 was also significantly related to graft loss at 1 month (OR 1.248, p=0.029; 95% CI 1.022-1.524). ALT on POD 3 showed a strong relation with graft loss at 2 weeks (OR 1.306, p=0.041; 95% CI 1.011-1.687) and 1 month (OR 1.474,p=0.020; 95% CI 1.062-2.046). OR are calculated for a rise of AST or ALT of 500 U/L).

Using cox regression analysis (univariate analysis), the hazard ratio for graft loss according to AST and ALT during POD 1 to 3 failed to show a significant prognostic value. Eg, a cut-off of 2000 U/L for AST on POD2 lacks a significant discriminative value as illustrated by the Kaplan-Meier curve (Log Rank test: p= 0.780) (Figure 3).

Patient survival

In contrast to graft survival, a significant relationship was observed using ROC analysis between AST on POD2 (AUC 0.890, p=0.022; 95%CI 0.737-1) and POD 3 (AUC 0.894, p=0.020; 95% CI 0.757-1) and ALT on POD 3 (AUC 0.869, p=0.030; 95% CI 0.668-1) with patient survival at 1 month. There was no significant predictive value for survival at 1 year.

Based on the youden-index, an optimal cut-off was derived from the AUC for AST on POD 2 of 1000 U/L, yielding a sensitivity of 100% and a specificity of 71%.

Using logistic regression (univariate analysis) AST on POD1 (OR 1.202, p=0.016; 95% CI 1.035-1.396), AST POD2 (OR 1.289, p=0.005; 95% CI 1.078-1.541) and ALT on POD 2 (OR 1.462, p=0.011; 95% CI 1.090-1.960) were significantly related with patient survival at 1 month but none was at 1 year. OR are calculated for a rise of AST or ALT of 500 U/L).

Using cox regression analysis (univariate analysis), only AST on POD 2 was predictive for patient death (HR 1.098, p=0.016; 95% CI 1.017-1.186). The lack of discriminative power, however based on the cut-off of 1000 U/L for AST on POD2 is illustrated by the Kaplan-Meier curve.

5.4.5 MULTIVARIATE COX REGRESSION ANALYSIS

Graft survival

Next, we built a multivariate Cox regression model taking into account the GlycoTransplantTest, AST POD 1, 2 and 3 and ALT on POD 1, 2 and 3. Only GlycoTransplantTest and AST on POD1 and 3 were predictive for graft loss during the first year after transplantation with a hazard ratio of respectively 8.003 (p<0.0001; 95% CI 3.00-21.347), 1.413 (p=0.007; 95% CI 1.101-1.813) and 0.297 (p=0.031; 95% CI 0.099-0.896).

When a new multivariate Cox regression model was built including GlycoTransplantTest, EAD (as defined by Olthoff[32] and thus including peak transaminases) and DRI, only the GlycoTransplantTest was independently related to graft loss (p=0.003).

Patient survival

A similar model was built regarding patient survival including GlycoTransplantTest (using the cut-off of -0.74), AST on POD 1, 2 and 3 and ALT on POD 1, 2 and 3. GlycoTransplantTest (HR 5.231, p=0.025; 95% CI 1.374-19.920) and ALT on POD1 (HR 0.998, p=0.048; 95% CI 0.997-1.000) were predictive for patient death.

In a model including GlycoTransplantTest, EAD and DRI, none of these parameters was significantly related to patient survival.

5.4.6 CORRELATION OF GLYCOTRANSPLANTTEST AND EAD

An intriguing question is whether the GlycoTransplantTest is not just a diagnostic marker of EAD, as EAD is also related with graft failure and patient death during the first year after liver transplantation. However, using Spearman's correlation analysis we found no correlation between the GlycoTransplantTest and EAD as defined by Olthoff[32] (R=.0.39; p=0.685).

5.5 DISCUSSION

Based on the simple measurement of 2 glycans in serum on day 7 after LT we can reliably predict the risk of graft loss and patient death during the first year after LT. This measurement was incorporated into a new biomarker, called the GlycoTransplantTest. The overall hazard ratio for graft loss during the first year after LT using this assessment was 7.222 (p<0.001;95% CI 2.352-22.182) using a cut-off above -0.81. As can be appreciated from the Kaplan Meier curve (Figure 4), the optimal performance was observed for the prediction of graft loss at 3 months with an odds ratio of 8.118 (p<0.0001; 95%CI 2.784-23.675).

This work adds to the increasing evidence that glycomic-based biomarkers reflect in a reliable way specific dysfunctions occurring in the liver[22,24,28,30,33–35]. Although 7 out of 13 peaks in the glycomic analysis were significantly altered in patients with a poor outcome, this information could be captured in a glycomic signature with only 2 single N-glycans, the increase of peak 3, an agalacto, core-alpha-1,6-fucosylated biantennary glycan (NG1A2F) and the decrease of peak 8, a triantennary

glycan (NA3). 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[25,36]. 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[37], infections or sepsis. Oweira et al. showed an independent association between postoperative inflammation after LT and graft loss and patient death[38]. This inflammatory response has been related to an increase of IL-6[39], IL-2R, IL-7, IP-10, MIG[37]. Also IL-8, CCL2 and CCL5 are upregulated in the early postoperative phase resulting from the Nf-kB pathway[37]. Interestingly, this study demonstrates that the increase of peak 3 (NG1A2F) is an elegant and robust marker of this inflammatory response.

In contrast to the undergalactosylated glycans, the decrease of NA3, a triantennary glycan, is hepatocyte-driven[25] 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[22]. The action of N-acetylglucosaminyltransferase V (GnT-V), involved in the formation of precursor glycans of NA3, might be diminished in favour 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[26]. 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[40]. Second, in a two-thirds partial hepatectomy model in rats, GnT-V activity was increased in hepatocytes and non-parenchymal cells during regeneration[41]. Possibly, the decreased levels of NA3 in patient with graft loss illustrate a lack of the required regeneration capacity after liver transplantation resulting in graft failure.

In this cohort, we confirmed the predictive role of peak transaminases during day 1 to 3 regarding graft loss and patient death, as reported earlier by other groups[12,15,31,4213]. However, in the same cohort, the prognostic value of the GlycoTransplantTest was superior to AST and ALT measurement. Furthermore, in a multivariate Cox regression model including the GlycoTransplanttest, EAD (as defined by Olthoff[32]) and DRI, only the GlycoTransplantTest was independently predictive of graft survival.

According to this analysis the GlycoTransplantTest might be an attractive tool in the management of patients with suboptimal graft function in the first week after liver transplantation. 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 monocentric cohort we showed the best prognostic

performance for graft survival with a single measurement of GlycoTransplantTest 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.

The main limitation is the monocentric character of this study and the current absence of external validation. To our knowledge however, this is the first biomarker that offers this high predictive value with an OR for graft loss at 3 months of 8.118 (p<0.0001; 95%CI 2.784-23.675).

The GlycoTransplantTest is measured using a routine DNA-sequencer and will shortly be implemented on less expensive and widely available high-throughput microfluidics CE platforms, which will make the technology accessible for routine clinical laboratories.

We conclude in line with previous reports of our group that have highlighted the value of glycomics-based biomarkers in liver disease[24,29,30,35], that a glycomic assessment of serum at day 7 after liver transplantation offers a reliable marker of graft function that is an independent predictor of graft survival within the first year after LT and is related to patient survival. Hence, it could be an attractive tool for guidance in decision making when a retransplantation is considered. The application of this technology on high-throughput microfluidics CE platforms could facilitate an easy implementation in clinical practice.

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Supplementary Table

Overview of glycomic alterations related to outcome. Only glycans with a significant association with outcome are shown. Significant associations are markerd in grey. Results are presented as p-values after logistic regression (univariate analysis). For the marker "Max value day 1 to 7" the highest value between day 1 and 7 was used.

	Peak 1			
	NGA2F			
	Max value day 1 to 7	Value Day 1	Value Day 3	Value Day 7
Patient survived 1 month	0.663	0.059	0.492	0.650
Patient survived 1 year	0.795	0.463	0.732	0.915
Organ survived 2 weeks	0.791	0.912	0.506	0.882
Organ survived 1 month	0.519	0.779	0.063	0.051
Organ survived 3 months	0.474	0.734	0.018	0.023
Organ survived 1 year	0.269	0.763	0.025	0.035
	Peak 3			
	NG1A2F			
	Max value day 1 to 7	Value Day 1	Value Day 3	Value Day 7
Patient survived 1 month	0.348	0.294	0.949	0.589
Patient survived 1 year	0.051	0.234	0.090	0.557
Organ survived 2 weeks	0.208	0.050	0.103	0.041
Organ survived 1 month	0.017	0.551	0.012	0.002
Organ survived 3 months	0.010	0.243	0.001	0.003
Organ survived 1 year	0.007	0.106	0.001	0.009
	Peak 5			
	NA2			
	Max value day 1 to 7	Value Day 1	Value Day 3	Value Day 7
Patient survived 1 month	0.065	0.379	0.248	0.093
Patient survived 1 year	0.968	0.395	0.752	0.492
Organ survived 2 weeks	0.840	0.720	0.248	0.316
Organ survived 1 month	0.061	0.014	0.020	0.791

	0.028	0.014	0.019	0.836
Organ survived 3 months	0.028	0.014	0.019	0.836
Organ survived 1 year	0.028	0.030	0.058	0.920

Peak 6

NA2F

	Max value day 1 to 7	Value Day 1	Value Day 3	Value Day 7
Patient survived 1 month	0.539	0.215	0.982	0.387
Patient survived 1 year	0.007	0.119	0.013	0.310
Organ survived 2 weeks	0.043	0.098	0.759	0.423
Organ survived 1 month	0.280	0.370	0.231	0.086
Organ survived 3 months	0.352	0.825	0.054	0.139
Organ survived 1 year	0.026	0.356	0.009	0.037

Peak 7

NA2FB

	Max value day 1 to 7	Value Day 1	Value Day 3	Value Day 7
Patient survived 1 month	0.563	0.115	0.304	0.295
Patient survived 1 year	0.396	0.849	0.043	0.250
Organ survived 2 weeks	0.856	0.674	0.453	0.268
Organ survived 1 month	0.878	0.311	0.533	0.471
Organ survived 3 months	0.796	0.617	0.274	0.121
Organ survived 1 year	0.377	0.825	0.024	0.015

Peak 8

NA3

	Max value day 1 to 7	Value Day 1	Value Day 3	Value Day 7
Patient survived 1 month	0.307	0.206	0.994	0.278
Patient survived 1 year	0.118	0.720	0.346	0.084
Organ survived 2 weeks	0.510	0.843	0.420	0.078
Organ survived 1 month	0.010	0.017	0.058	0.008
Organ survived 3 months	0.010	0.118	0.042	0.006
Organ survived 1 year	0.004	0.123	0.045	0.004

Peak 9

NA3FB

	Max value day 1 to 7	Value Day 1	Value Day 3	Value Day 7
Patient survived 1 month	0.600	0.608	0.810	0.742
Patient survived 1 year	0.613	0.199	0.412	0.728
Organ survived 2 weeks	0.603	0.578	0.395	0.817
Organ survived 1 month	0.090	0.050	0.032	0.143
Organ survived 3 months	0.128	0.117	0.014	0.411
Organ survived 1 year	0.181	0.046	0.014	0.935
Peak 9bis **NA3Fc**				
	Max value day 1 to 7	Value Day 1	Value Day 3	Value Day 7
Patient survived 1 month	0.128	0.207	0.527	0.043
Patient survived 1 year	0.521	0.730	0.786	0.383
Organ survived 2 weeks	0.084	0.475	0.304	0.030
Organ survived 1 month	0.014	0.027	0.497	0.037
Organ survived 3 months	0.045	0.312	0.956	0.018
Organ survived 1 year	0.038	0.439	0.944	0.052
Peak 10 **NA3FBc**				
	Max value day 1 to 7	Value Day 1	Value Day 3	Value Day 7
Patient survived 1 month	0.633	0.098	0.739	0.793
Patient survived 1 year	0.554	0.254	0.491	0.936
Organ survived 2 weeks	0.406	0.629	0.264	0.164
Organ survived 1 month	0.036	0.020	0.043	0.046
Organ survived 3 months	0.057	0.094	0.043	0.099
Organ survived 1 year	0.041	0.037	0.030	0.054
Peak 11 **NA4**				
	Max value day 1 to 7	Value Day 1	Value Day 3	Value Day 7
Patient survived 1 month	0.669	0.121	0.681	0.904
Patient survived 1 year	0.840	0.038	0.364	0.174

Organ survived 2 weeks	0.903	0.647	0.325	0.731
Organ survived 1 month	0.161	0.053	0.030	0.516
Organ survived 3 months	0.148	0.080	0.014	0.739
Organ survived 1 year	0.127	0.011	0.010	0.739

Relevant results of ROC curve analysis for the prognostic value of GlycoTransplantTest

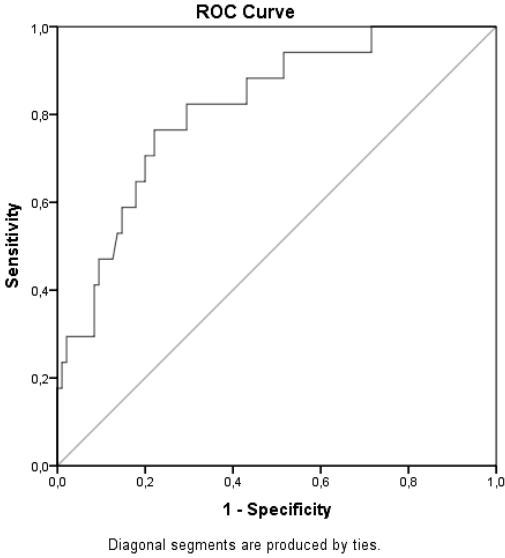**Graft loss at 2 weeks:** AUC = 0.824 (p=0.028; 95%CI 0.597-1.000)
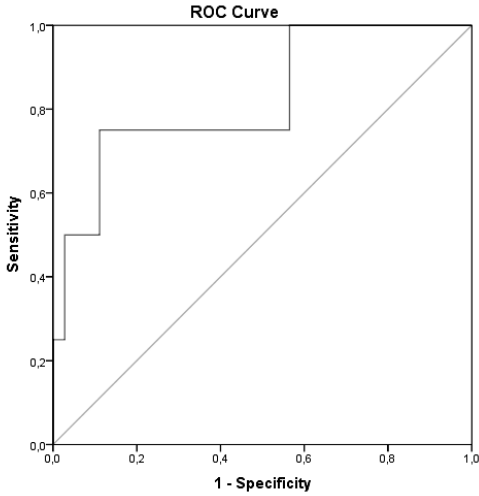
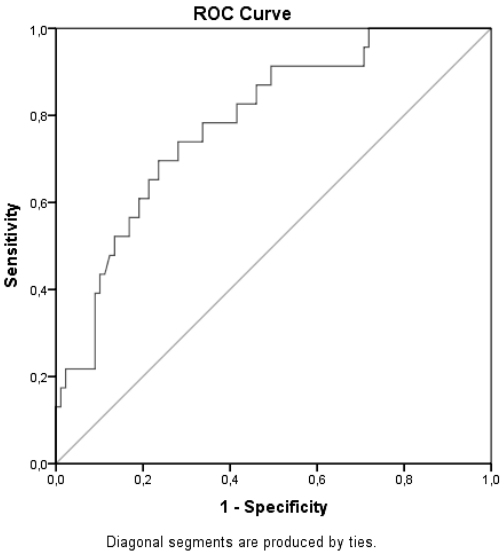**Graft loss at 1 month:** AUC =0.810 (p<0.0001; 95%CI 0.686-0.934)
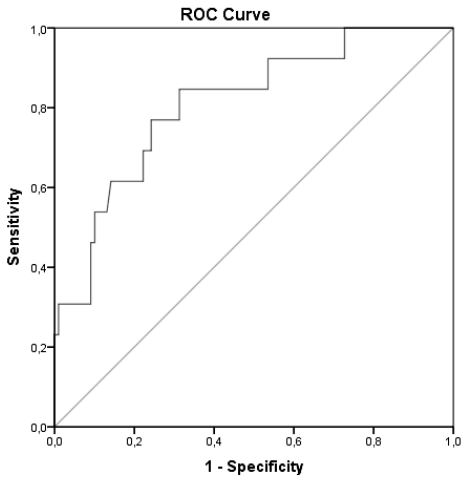
Diagonal segments are produced by ties.

Graft loss after 3 month : AUC = 0.816 (p<0.0001; 95%CI 0.712-0.920)

Diagonal segments are produced by ties.

Graft loss after 1 year : AUC = 0.784 (p<0.0001; 95%CI 0.684-0.884)

Diagonal segments are produced by ties.

Patient death at one month : AUC = 0.817 (NS)
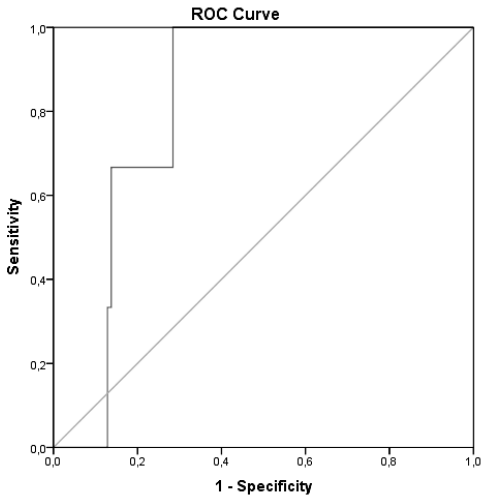
Patient death at one year: AUC = 0.702 (p=0.028; 95%CI 0.566-0.838).
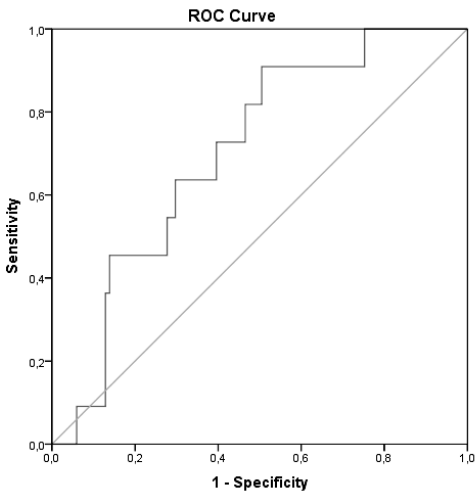
VI. The potential of glycomics-based biomarkers to assess the development of acute cellular rejection after liver transplantation

Assessment of a glycomic signature in serum as a non invasive diagnostic marker for acute cellular rejection after liver transplantation

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6.1 ABSTRACT

Non-invasive biomarkers for acute cellular rejection (ACR) are an unmet medical need. In this exploratory study we assessed whether specific glycomic changes could be observed in the serum of liver transplant patients during the development of ACR (confirmed by liver biopsy). Serum glycomic analysis was performed as described before using DSA-FACE. In a cohort of 108 liver transplant patients, 38 ACR episodes were identified. The glycomic profiles were compared between these episodes and non-ACR serum samples.

A significant increase of NA2F (a bigalacto, core-alpha-1,6-fucosylated biantennary glycan) and a reciprocal significant decrease of NA2 (a bigalacto biantennary glycan) was observed in ACR serum samples compared to non-ACR serum samples. However, the clinical relevance of this finding is unclear, as there was an important overlap between both groups. However, these data provide a first proof-of-concept that (subtle) glycomic alterations are present in serum during ACR development after liver transplantation.

6.2 INTRODUCTION

A non-invasive diagnostic marker for ACR after LT is an unmet medical need and liver biopsy remains mandatory for the confirmation of ACR[1]. We explored whether specific glycoalterations are related to the development of ACR after liver transplantation.

6.2 METHODS AND MATERIALS

6.2.1 DESIGN

This was a monocentric prospective cohort study at Ghent University Hospital. All patients who underwent liver transplantation in the Liver Transplant Unit of Ghent University Hospital (Belgium) between 1 December 2012 and 31 December 2014 were eligible.

From every patient one serum sample was collected in the 24 hour timeframe before liver transplantation. After liver transplantation daily serum samples were collected during 14 days, and after 1,3,6 and 12 months after liver transplantation. Supplementary serum samples were taken at the moment of suspicion of ACR, as characterized by an increase of liver enzymes. ACR was

confirmed and graded by liver biopsy examination according to the Banff staging system[2]. CMV infection was assessed by CMV PCR measurement in serum.

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 outcome.

6.2.2 GLYCOMIC ANALYSIS

Five microliter of serum were processed according to the in-solution deglycosylation method described by Vanderschaeghe et al.[3]. 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[4]. The result of this analysis is a total desialylated serum protein electropherogram, which consists of 13 peaks. Each peak represents a well-identified glycan. 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.

6.2.3 STATISTICS

Statistical analysis was performed using IBM©SPSS©Statistics Version 22.0. The relative abundance of every peak was compared between ACR episodes and non-ACR episodes using Mann Whitney U test. A general linear mixed model was built that compared the relative abundance of every peak during ACR compared to non-ACR samples.

6.2.4 ETHICS

The study conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the Ghent University Hospital ethics committee.

6.3. Results

6.3.1 Patient characteristics

In this analysis, the results of 108 consecutive patients were collected. The baseline characteristics are summarized in table 1.

Table 1. Baseline characteristics of the liver transplant patients (n=113).

		Whole Cohort	Patients without ACR	Patients with ACR	
		Number (SD)	**Number (SD)**	**Number (SD)**	
Mean Age		50.65 (13.21)	53.2 (15.74)	50.39 (13.46)	p=0.677
Sex	Male	61	41	20	p=0.506
	Female	52	34	18	

Acute cellular rejection was diagnosed in 38 patients and confirmed on liver biopsy examination. ACR grade 1 was found in 22 patients, grade 2 in 14 patients and grade 3 in 2 patients.

Table 2. ACR episodes and CMV reactivation episodes

	Incidence	Percentage
ACR grade 1	22	20.37%
ACR grade 2	14	12.96%
ACR grade 2	2	1.86%
CMV reactivation	38	35.19%

6.3.2 Glycomics in ACR

We compared the mean levels of every single glycan during ACR episodes and non-ACR episodes amongst all patients. All ACR episodes were pooled for this analysis. The results are summarized in table 3. CMV reactivation episodes were excluded for this analysis. Two glycans showed significant alterations during ACR episodes compared to non-ACR episodes. The relative abundance of NA2F, a bigalacto, core-alpha-1,6-fucosylated biantennary glycan, was significantly increased during ACR

episodes, whereas the relative abundance of NA2, a bigalacto biantennary glycan, was decreased during ACR episodes (Figure 1).

Table 3. Comparison of mean values of glycan-abundance between rejection and non-rejection episodes (Mann Whitney U test).

|	ACR (SD)	No ACR (SD)	p-value
NGA2F	5,31 (3.42)	6,07 (3.82)	0.311
NGA2FB	1.05 (1.08)	1.09 (0.80)	0.482
NG1A2F (peak 3)	2.67 (1.44)	2.89 (1.60)	0.642
NG1A2F (peak 4)	3.73 (1.77)	4.20 (2.00)	0.255
NA2	41.86 (15.50)	45.13 (13.30)	**0.042**
NA2F	12.30 (5.06)	11.76 (4.28)	**0.033**
NA2FB	4.31 (2.23)	4.17 (2.42)	0.193
NA3	7.34 (3.59)	8.06 (3.52)	0.320
NA3FB	5.18 (3.24)	5.28 (3.00)	0.770
NA3FC	0.928 (0.54)	0.83 (0.49)	0.142
NA3FBC	2.49 (1.38)	2.63 (1.36)	0.789
NA4	1.43 (0.93)	1.40 (0.80)	0.669
NA4FB	0.59 (0.47)	0.55 (0.42)	0.686
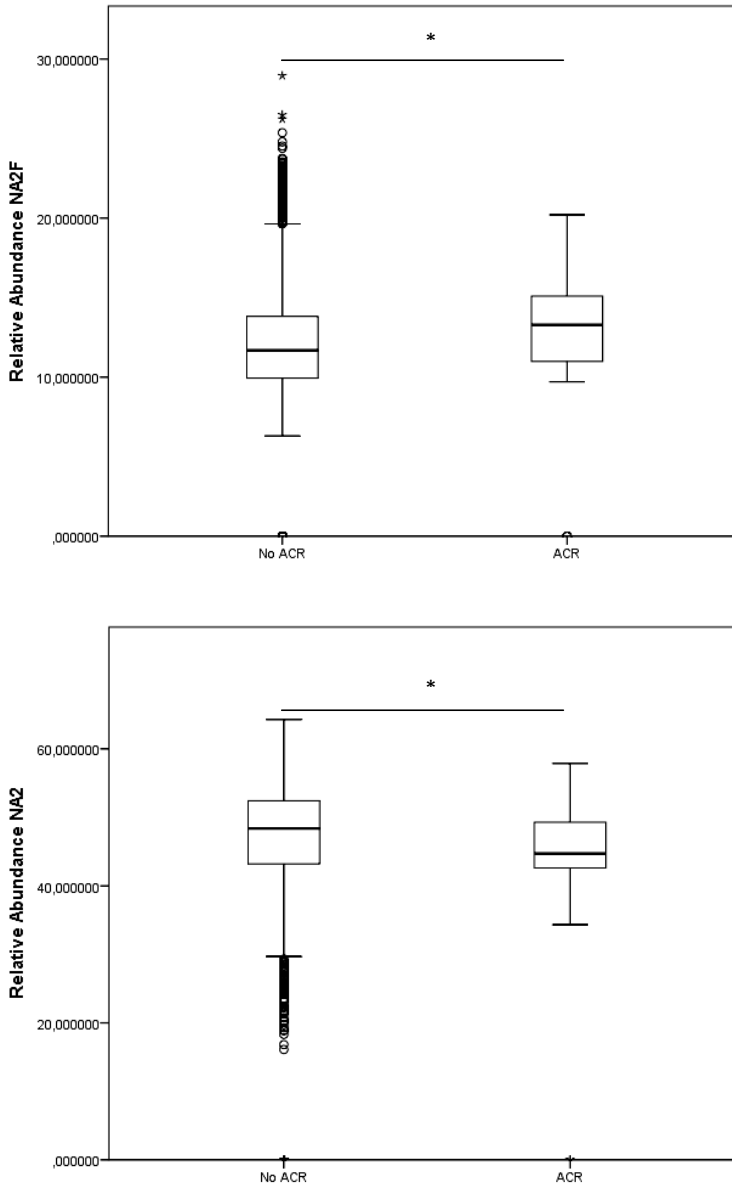
Figure 1. Relative abundance of NA2F was significantly increased during ACR episodes, whereas the relative abundance of NA2 was significantly decreased. *p=<0.05

6.3.3 GLYCOMICS IN CMV REACTIVATION

We compared the mean levels of every single glycan during CMV reactivation episodes and non-CMV reactivation episodes amongst all patients. All ACR episodes were excluded for this analysis. The results are summarized in table 4. One glycan, NA3FB (a branch fucosylated tri-antennary glycan) showed a significant increase during CMV reactivation (Figure 2).

Table 4. Comparison of mean values of glycan-abundance between CMV reactivation and non reactivation episodes (Mann Whitney U test).

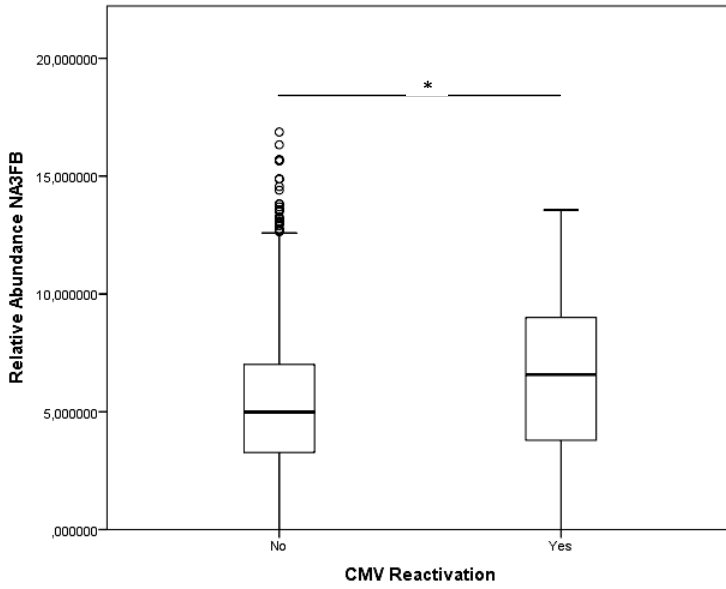|	CMV reactivation (SD)	No CMV reactivation (SD)	p-value
NGA2F	5.35 (2.85)	6.08 (3.83)	0.420
NGA2FB	1.00 (0.83)	1.09 (0.80)	0.289
NG1A2F (peak 3)	2.63 (1.34)	2.89 (1.60)	0.516
NG1A2F (peak 4)	3.72 (1.95)	4.21 (2.00)	0.071
NA2	43.46 (14.07)	45.15 (13.29)	0.206
NA2F	11.32 (4.18)	11.77 (4.28)	0.790
NA2FB	4.19 (2.59)	4.17 (2.42)	0.856
NA3	8.03 (2.60)	8.036 (3.52)	0.938
NA3FB	6.61 (3.72)	5.26 (2.91)	**0.013**
NA3FC	0.80 (0.49)	0.834 (0.49)	0.730
NA3FBC	2.72 (1.45)	2.63 (1.36)	0.747
NA4	1.59 (0.95)	1.36 (0.80)	0.130
NA4FB	0.65 (0.48)	0.55 (0.42)	0.163

Figure 2. Relative abundance of NA3FB was significantly increased during CMV reactivation episodes. *p=<0.05

6.3.4 GENERAL LINEAR MIXED MODEL FOR ACR AND CMV REACTIVATION

A general linear mixed model could not confirm the relevance of these glycoalterations during ACR and CMV in this integrated approach (table 5).

Table 5: Comparison of the relative abundance of the difference glycans during the ACR episodes. A general linear mixed model was used. P-values are shown.

	ACR Grade 1	ACR Grade 2	ACR Grade 3	CMV reactivation
NGA2F	0.749	0.990	0.704	0.686
NGA2FB	0.948	0.907	0.921	0.812
NG1A2F (peak 3)	0.694	0.873	0.794	0.734
NG1A2F (peak 4)	0.602	0.970	0.619	0.605
NA2	0.538	0.925	0.610	0.782
NA2F	0.881	0.772	0.772	0.816
NA2FB	0.893	0.825	0.887	0.988
NA3	0.721	0.952	0.644	0.990
NA3FB	0.615	0.701	0.935	0.318
NA3FC	0.829	0.918	0.691	0.901
NA3FBC	0.798	0.983	0.782	0.870
NA4	0.755	0.730	0.884	0.525
NA4FB	0.677	0.652	0.862	0.833

6.4. DISCUSSION

In the introduction of this booklet, we included a review that highlights the medical need for non-invasive diagnostic markers for ACR after liver transplantation. Hoping to answer this question, we studied the behavior of glycans during the development of acute cellular rejection in this exploratory analysis. When comparing the mean values of several glycans during ACR and non-ACR episodes, a significant increase was observed of the relative abundance of NA2F (a bigalacto, core-alpha-1,6-fucosylated biantennary glycan) and a reciprocal significant decrease of NA2 (a bigalacto biantennary

glycan). These changes were not apparent during CMV reactivation, another frequent cause of increased transaminases after liver transplantation. However, in general linear mixed model this observation could not be confirmed. Furthermore, as can be appreciated from figure 1, there is an important overlap between both ACR and non-ACR episodes.

Increased alpha-1,6-fucosylation has been observed in cholestatic liver diseases[5]. This fucosylation of N-linked glycans within polarized hepatocytes directs glycoproteins to the basolateral surface and into the bile canaliculli. As a consequence, alpha-1,6- fucosylated glycoforms are normally rare in the blood and are enriched in the bile. Thus, if liver cells become depolarized, the alpha1–6 fucosylated glycoforms rise in abundance in the blood. The inflammatory changes observed during ACR might lead to depolarization of the hepatocyte and result in the increase of NA2F.

Acute cellular rejection is often diagnosed in an early stage and the majority of the ACR episodes in this cohort were mild to moderate. This means that the inflammatory changes are modest, what might explain the subtle changes in the whole serum protein glycomic signature.

Although this finding is not useful for clinical practice at this moment, we need to explore this more in detail. First of all, we are including more patients in order to have more patients and more ACR and CMV reactivation events. This will allow us to confirm this observation and to study sequential measurements of the glyco-alterations before and during the treatment of ACR in individual patients. An approach using the delta of these glycoalterations in single patients during ACR has not yet been performed.

In conclusion, only subtle changes in the glycomic signature were observed during the development of ACR. However, we believe that the inclusion of more patients with ACR will clarify the potential role of glycomics in the diagnosis of ACR. From a pathophysiological point of view, it could make sense that a specific glycomic signature for ACR can be found.

6.5 REFERENCES

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VII. The potential of glycomics-based biomarkers to assess outcome in acute liver failure

Serum N-glycans are predictive of transplant free survival during paracetamol-induced acute liver injury : a proof-of-concept

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7.1. ABSTRACT

Acetaminophen-induced acute liver failure (APAP-induced ALF) is a rare but life-threatening illness. A small proportion of these patients will require urgent liver transplantation. The decision to list for transplantation is guided by clinical criteria, e.g. the Kings' college or Clichy criteria. Serum N-glycans (glycomics) are altered in several liver diseases but have not been assessed as prognostic markers in ALF. The first goal of this work was the serial assessment of the whole serum glycomic profile during the evolution of APAP-induced acute liver injury. The second goal was an evaluation of the potential of serum glycomics as prognostic markers in APAP-induced acute liver injury.

During APAP-induced acute liver injury and the recovery of acute liver injury consistent glycomic changes are observed, reflecting the regeneration process. Recovery of acute liver injury was characterized by an increased fucosylation and increased branching of sugar structures. In this cohort of 11 patients, a distinct glycomic profile was present at admission in patients with transplant-free survival (p=0.036). In contrast, patients requiring liver transplantation showed an increased undergalactosylation, a sign of major inflammation, at admission.

In conclusion, the recovery of APAP-induced acute liver injury is characterized by increased fucosylation and increased branching. Furthermore, glycomic assessment of serum at baseline can predict transplant-free survival in APAP-induced ALF.

7.2. INTRODUCTION

Acute liver failure (ALF) is a rare but life-threatening illness that occurs in fewer than 10 cases per million persons per year. It is most often observed in previously healthy young adults[1]. ALF was originally named "fulminant hepatic failure" and defined as "a severe liver injury, potentially reversible in nature and with onset of hepatic encephalopathy within 8 weeks of the first symptoms in the absence of pre-existing liver disease"[2]. In the Western world acetaminophen (N-acetyl-p-aminophenol; APAP; paracetamol) overdose, is the leading cause of ALF[34]. If the antidote N-acetylcysteine is administered within 10 hours of ingestion, outcome is excellent in the majority of patients[5,6].

The main challenge in the care for patients with APAP-induced ALF is the identification of patients that will not survive with medical therapy alone. Early identification of patients that will require liver transplantation is of utmost importance[7].

Various prognostic models have been developed, mainly based on historical patient cohorts treated without liver transplantation. The most well-known models are the King's college criteria which include specific criteria for APAP-induced ALF[8] and the Clichy[9] criteria, although the latter was initially developed for ALF caused by viral hepatitis. Both have been externally validated[10,11,12]. Key factors in these models are age, development of encephalopathy and coagulopathy. King's college criteria also include acidosis and renal failure. These models are daily used for clinical decision making. However these criteria have clinically acceptable specificity but a more limited sensitivity[11,12]. The need for improved identification of candidates for transplantation is clear[1].

We have previously shown that "glycomics"-based biomarkers based on profiling the N-glycans from the total serum protein using capillary electrophoresis (CE), are useful for fibrosis monitoring[13] and diagnosis of cirrhosis[14] in patients with chronic liver diasease, and for the identification of patients with non alcoholic steatohepatitis among patients with non alcoholic fatty liver disease[15,16]. Indeed, glycomics-based biomarkers are particularly attractive for use in liver diseases, as hepatocyte-secreted glycoproteins and immunoglobulin G are the dominant glycosylated serum proteins[17–19].

In this study, an assessment was performed of the alterations of the serum protein N-glycomic profile during the development and recovery of paracetamol-induced acute liver injury and ALF. Furthermore we investigated whether the serum protein N-glycomic profile is predictive for outcome in paracetamol-induced ALF.

7.3.   MATERIALS AND METHODS

This single center study was performed in the department of Gastroenterology and Hepatology of Ghent University Hospital (Belgium), which is a referral unit for liver transplantation.

7.3.1 STUDY COHORT AND STUDY DESIGN

This exploratory cohort consisted of 11 patients. Patients were eligible upon admission for APAP intoxication with elevated liver enzymes (>2x ULN) and decrease of prothrombin time below 60%. From admission on, a daily serum sample was taken until normalization of coagulation or liver transplantation. Serum samples were prospectively collected between 1 June 2012 and 31 December 2015 and stored at minus 20°C.

7.3.2 GLYCOMIC ANALYSIS

N-glycan analysis was performed using DSA-FACE as described before[19]. In summary, five microliter of serum was processed according to the in-solution deglycosylation method described by Vanderschaeghe et al.[19]. 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 removing the terminal sialic acid residues, the glycans were labeled with 8-aminopyrene-1,3,6-trisulphonic acid and analyzed using an ABI3130 DNA sequencer as described[20]. The result of this analysis is a total serum protein electropherogram, which consists of 13 peaks (Figure 1). Each peak represents a well-identified glycan[21]. The numerical height of every peak is quantified and normalized to the sum of all peak heights, thus represented as a percentage of total peak height.

For every serum sample, the relative abundance of 13 individual N-glycans was analysed (Figure 1). The molecular structure of every glycan has been elucidated and extensively described in former publications [14,21].

The relative abundance of every glycan was calculated and the day-by day evolution of glycans was studied in order to observe the evolution of these glycans during ALF up to 7 days after admission.

7.3.3 STATISTICAL ANALYSIS

Statistical analysis was performed using IBM[©]SPSS[©]Statistics Version 22.0. Mann Whitney U test was used when mean levels of biomarkers were compared between patients that recovered spontaneously and those who needed liver transplantation. Statistical significance was set at the alpha level = 0.05.

7.3.4 ETHICS

The study adheres to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the Ghent University Hospital ethics committee. Informed consent was obtained.
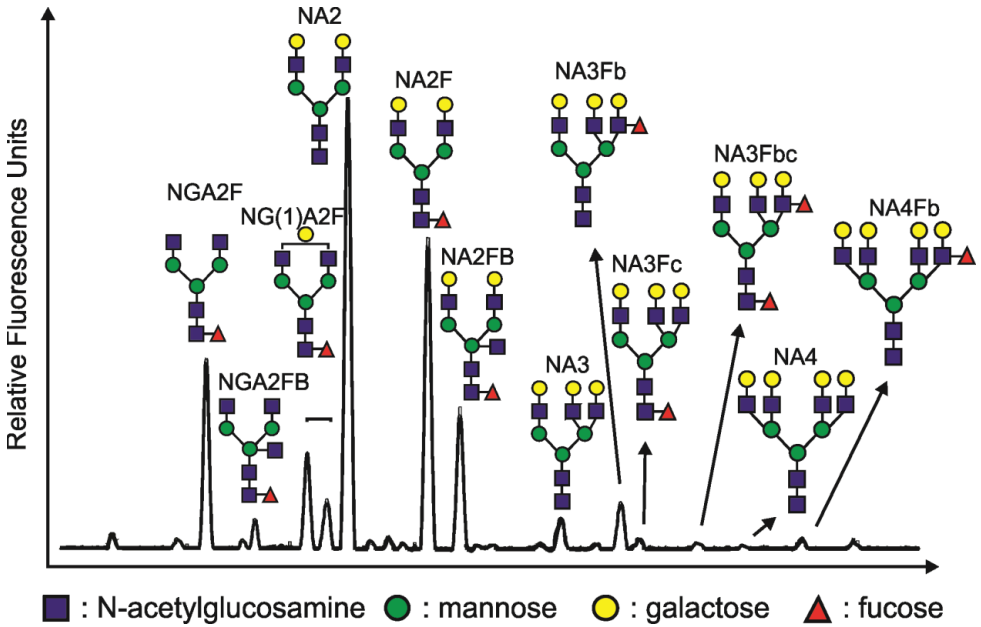
Fig 1. The structures of the N-glycan peaks in a human total serum sample. The structures of the N-glycan peaks in a human total serum sample as obtained using capillary electrophoresis yields 13 peaks. From left to right : Peak 1 is an agalacto, core-alpha-1,6-fucosylated biantennary (NGA2F), peak 2 is an agalacto, core-alpha-1,6-fucosylated bisecting biantennary (NGA2FB), peak 3 and peak 4 are monogalacto, 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 9bis is the core-alpha-1,6-fucosylated triantennary glycan NA3Fc, 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.

7.4. RESULTS

7.4.1 PATIENT CHARACTERISTICS

Eleven patients were included in this study with APAP-induced acute liver injury. All patients were treated with intravenous N-acetylcystein and maximal supportive care. Patients were admitted to an intensive care unit if appropriate. Demographic and clinical characteristics of the patients in this cohort are described in table 1. Two of those patients fulfilled the King's college criteria for liver transplantation for APAP-induced ALF[11] and underwent urgent liver transplantation.

Table 1. Summary of patient characteristics.

Patient Number	Age	Sex	Regular alcohol intake	Peak AST (U/l)	Peak ALT (U/l)	Lowest PT (%)	Encefalopathy	Outcome	King's college criteria for liver transplantation
1	29	M	Yes	9311	4582	32	No	Recovery	No
2	44	W	Yes	12359	8297	28	No	Recovery	No
3	48	W	No	3260	4001	45	No	Recovery	No
6	24	M	Yes	8481	5710	28	No	Recovery	No
7	56	W	Yes	5116	6136	12	No	Recovery	No
8	56	M	No	1247	727	45	No	Recovery	No
9	45	M	Yes	33414	5742	12	No	Recovery	No
14	57	M	No	401	2302	52	No	Recovery	No
15	30	W	No	11942	11085	16	No	Recovery	No
12	41	W	Yes	22848	8604	9	Yes	LT	Yes
18	21	W	No	7091	3600	14	Yes	LT	Yes

Legend : M: male; W: woman; LT : liver transplantation

7.4.2 SERIAL ASSESSMENT OF GLYCOMICS DURING ALF

Serial serum samples were available for the 9 patients with a spontaneous recovery. The evolution of the relative abundance of every glycan for these 9 patients is summarized in Figure 2. Both patients that underwent urgent liver transplantation had only one serum sample available.

All glycans of all patients on the same time point from admission were integrated in a scatter plot in order to reveal underlying trends during the recovery of acute liver injury (Figure 3). The first 4 peaks
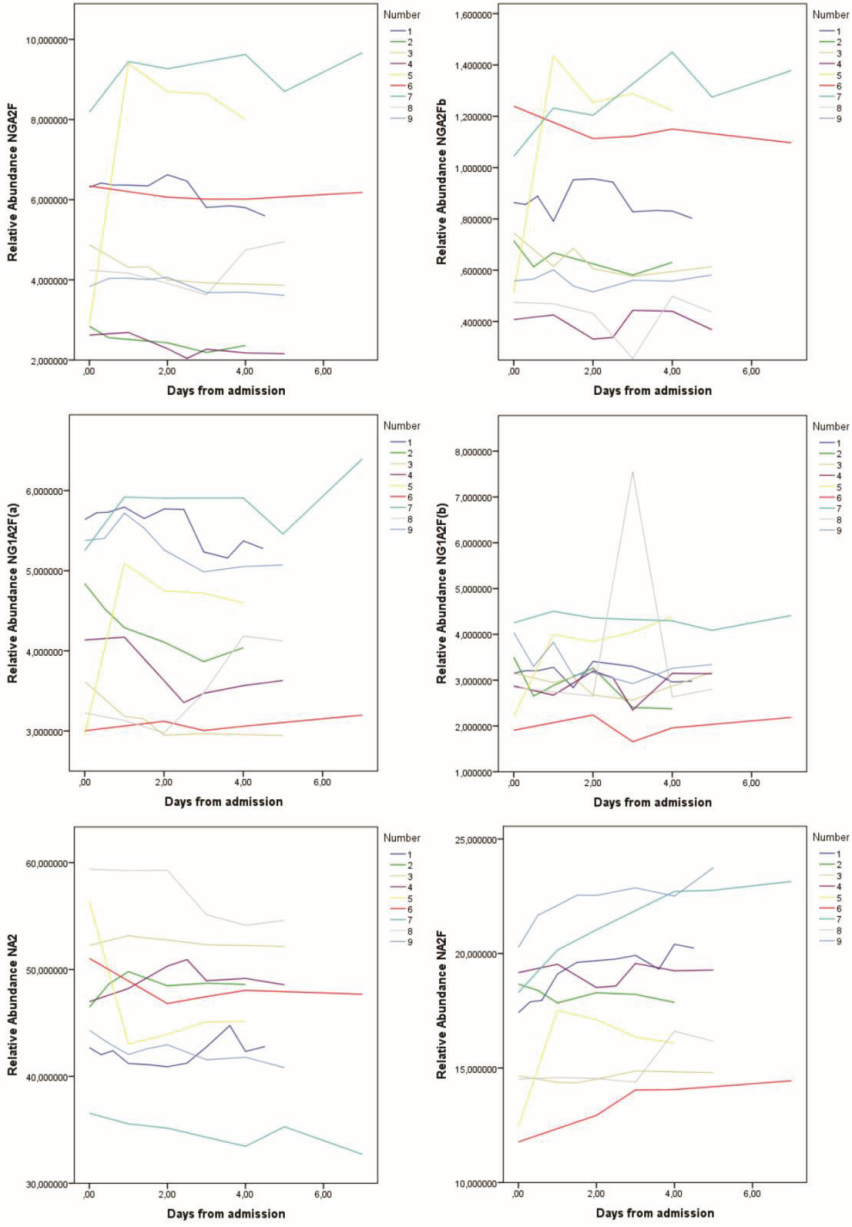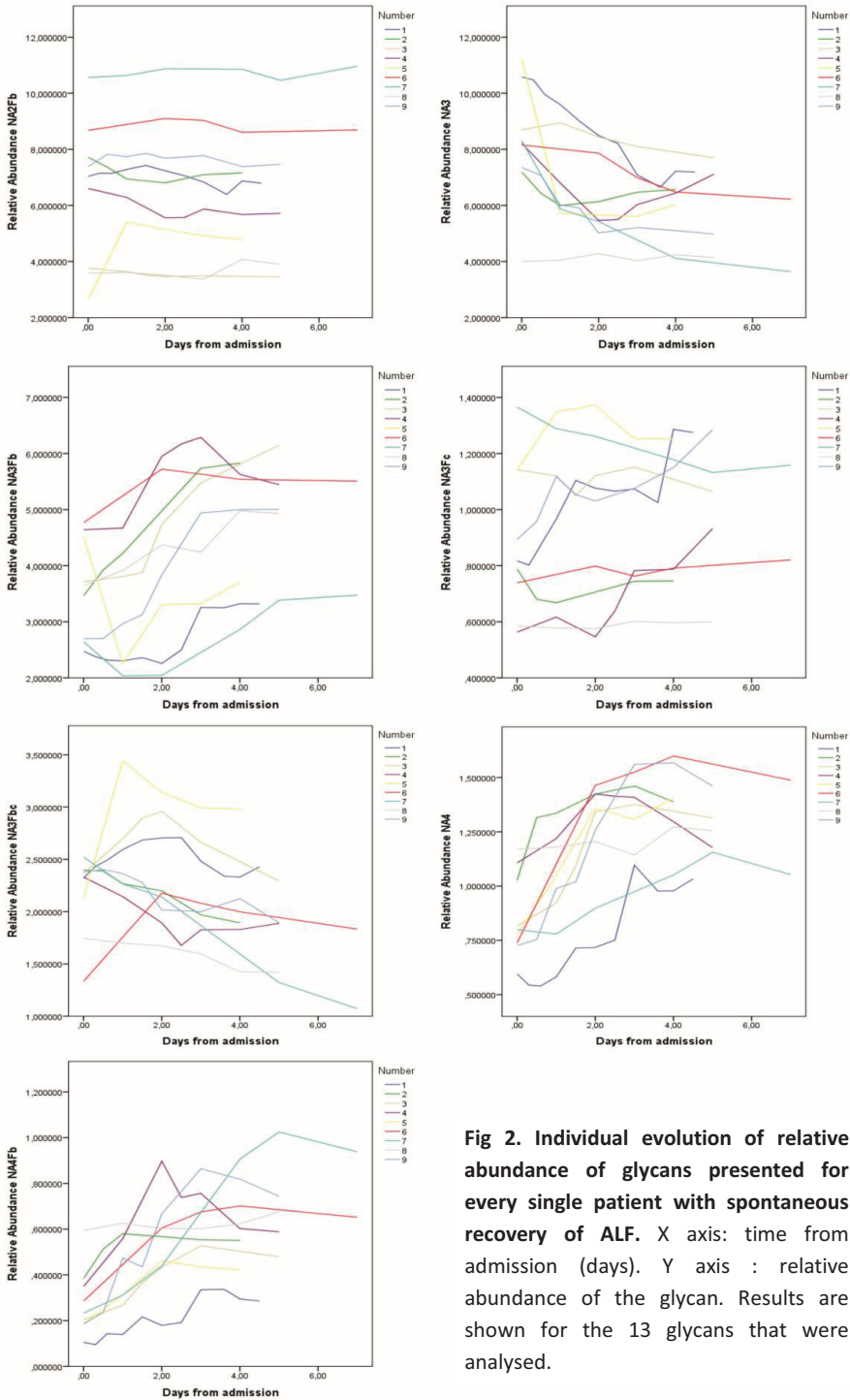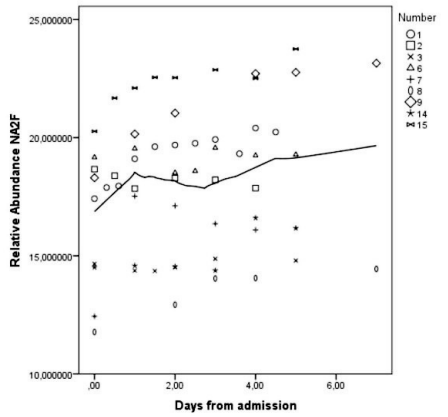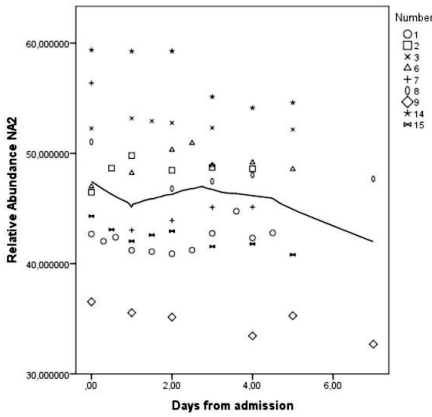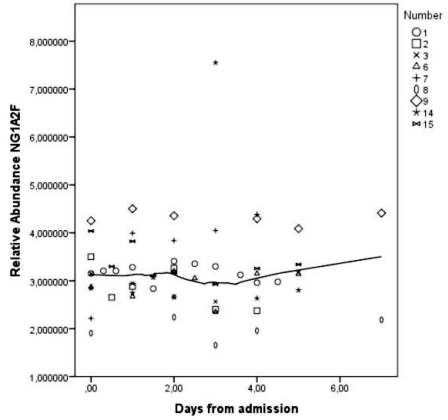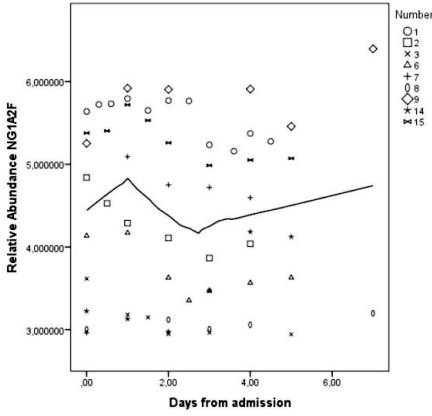
Fig 2. Individual evolution of relative abundance of glycans presented for every single patient with spontaneous recovery of ALF. X axis: time from admission (days). Y axis : relative abundance of the glycan. Results are shown for the 13 glycans that were analysed.
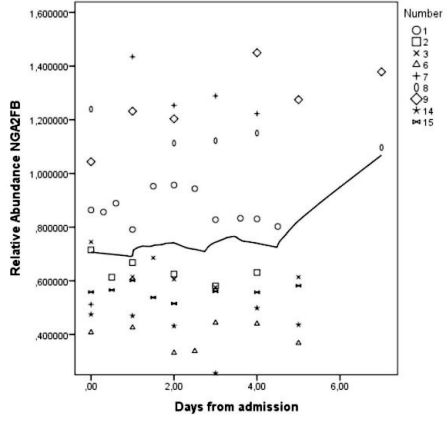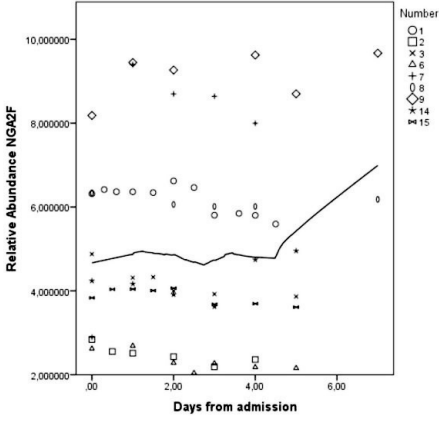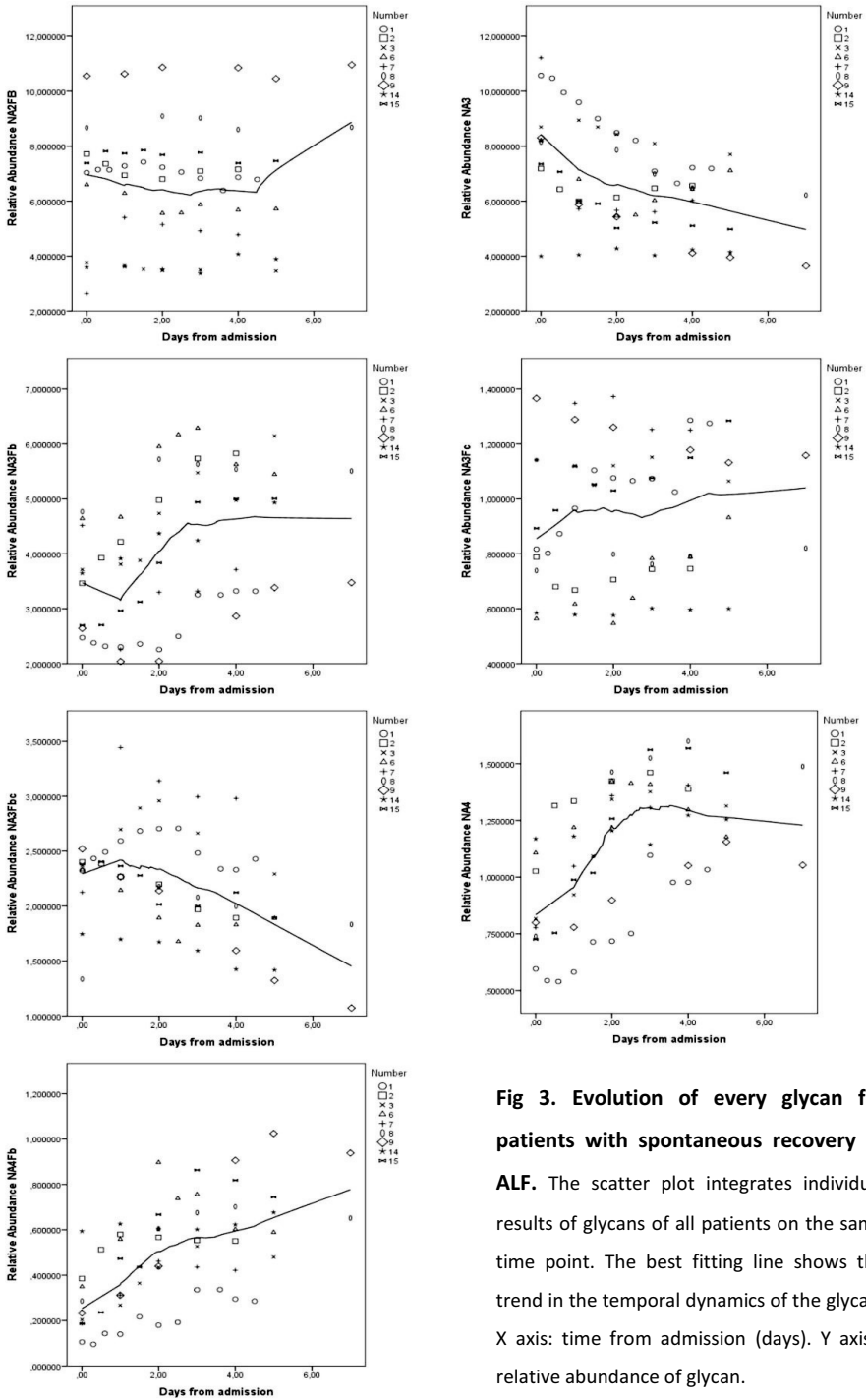
Fig 3. Evolution of every glycan for patients with spontaneous recovery of ALF. The scatter plot integrates individual results of glycans of all patients on the same time point. The best fitting line shows the trend in the temporal dynamics of the glycan. X axis: time from admission (days). Y axis : relative abundance of glycan.

are NGA2F,NGA2FB and NG(1)A2F (peak 3 and 4 are isomers of the same sugar structure). 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 monogalacto, core-alpha-1,6-fucosylated biantennary structures (NG1A2F). Throughout recovery, these glycans first show a stable or even maybe a downward trend, especially in peak 3. The fifth peak, the bigalacto biantennary glycan NA2, showed a stable trend. Peak 6, known as NA2F, a bigalacto, core-alpha-1,6-fucosylated biantennary glycan, showed an upward trend. Peak 7, the bigalacto, core-alpha-1,6-fucosylated bisecting biantennary glycan NA2FB, showed a stable trend. Peak 8, the triantennary glycan NA3, showed a downward trend. Peak 9, the branching alpha-1,3-fucosylated triantennary glycan NA3Fb and peak 9bis, the core-alpha-1,6-fucosylated triantennary glycan NA3Fc , trended upwards. Peak 10, the branching alpha-1,3-fucosylated and core alpha-1,6-fucosylated triantennary glycan NA3Fbc, showed a downward trend. The two last peaks, NA4 (a tetra-antennary glycan) and NA4Fb (branching alpha-1,3-fucosylated tetra-antennary glycan) showed an upward trend.

In conclusion, the most significant evolutions during the recovery of acute liver injury in patients not receiving liver transplantation are, compared to the baseline value :

1/ A possible moderate decrease of undergalactosylated sugar structures (especially peak 3)

2/ An increase of NA2F and NA2FB, both core-fucosylated biantennary glycans

3/ A marked and consistent increase of the complex branch fucosylated triantennary sugar NA3Fb and the tetra-antennary sugars NA4 and NA4Fb

7.4.3 PREDICTIVE VALUE OF GLYCANS FOR OUTCOME IN ACUTE LIVER INJURY

We compared the relative abundance of serum N-glycans in the first available serum sample upon admission, considering that in patients with ALF a rapid decision must be made to list the patient for liver transplantation or not.

The relative abundance of 4 glycans (both isomers of NG(1)A2F, NA2FB and NA3Fb) was significantly different at admission between patients that underwent liver transplantation and those who did not (p=0.036) as shown in Figure 4. The relative abundance of the NG(1)A2F isomers and NA2FB was increased at baseline in both patients undergoing liver transplantation compared to those who recovered. The relative abundance of NA3Fb was decreased at baseline in those patients in need of liver transplantation.
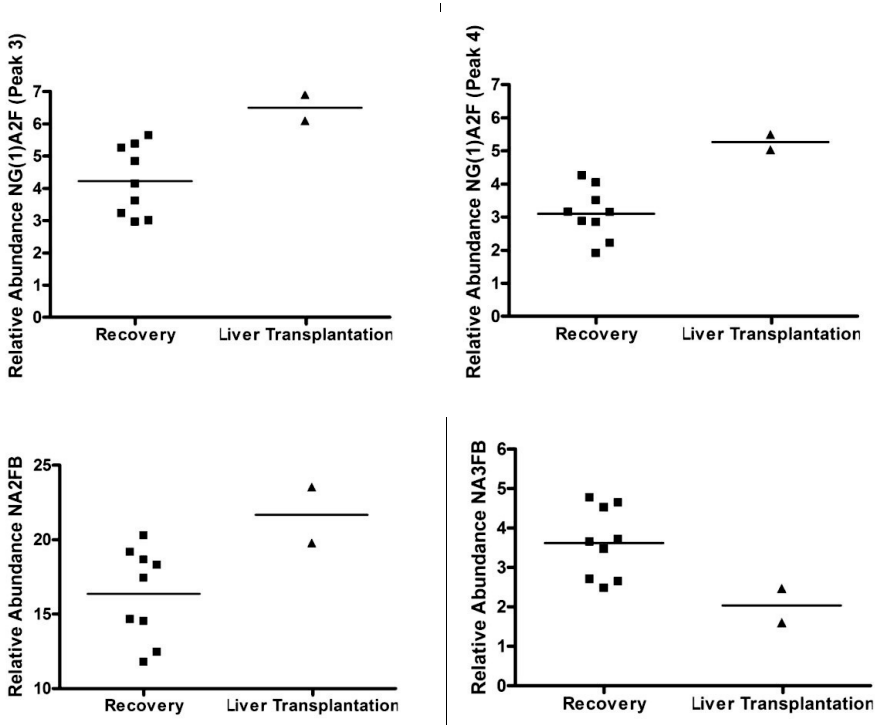
Fig 4. The relative abundance of NG(1)A2F (peak3), NG(1)A2F (peak4), NA2FB at baseline in patients with ALF with spontaneous recovery is significantly lower than in patients requiring liver transplantation (p=0.036). The relative abundance of NA3FB at baseline in patients with ALF with spontaneous recovery is significantly higher than in patients requiring liver transplantation (p=0.036). Groups were compared using Mann Withney U test.

7.5 DISCUSSION

This work describes the temporal dynamics of serum N-glycans in patients with APAP-induced acute liver injury and the potential role of serum N-glycans (glycomics) in the prediction of outcome in APAP-induced acute liver injury.

It is well established now that the measurement of serum N-glycans in whole serum is altered in underlying liver disease. During the last years our group contributed largely in the development of glycomics-based biomarkers that support this concept[14,17,20,22,23]. This led to the development of glycomics-based biomarkers for liver fibrosis[19], cirrhosis[14], non alcoholic steatohepatitis[15] and hepatocellular carcinoma (HCC)[24].

In contrast to our former work that studied "stable" liver disease states, this work focused on dynamic alterations during a rapidly and dramatically evolving liver disease *by excellence*, acute liver failure. Glycomic analysis of daily serum samples in patients with APAP-induced ALF showed consistent glycomic alterations during the evolution and recovery of ALF. The first finding is a trend towards a moderate *decrease of the undergalactosylated sugar structures* (peak 1-4). We and others formerly showed that undergalactosylation in the whole serum N-glycome is caused by undergalactosylation of immunoglobulins and not by liver derived proteins[19,25]. The development of APAP-induced ALF is characterized by the development of a complex inflammatory response, regulated by an interplay of different immune cells[26]. For example in mice, total leukocytes in the liver increases about threefold 18 hours after APAP injection[27]. The increased undergalactosylation reflects this important inflammatory response in ALF, especially in the patients who were in need of a LT, as these patients might show a more pronounced inflammatory reaction.

The second finding is a decrease of the non-fucosylated triantennary glycan NA3 and *an increase of the fucosylated biantennary glycans NA3FB and NA3FBc.* This finding is consistent with a report from 1990 from the group of King's college London that found increased fucosylation of several serum proteins in fulminant liver failure and patients with hepatocellular carcinoma[28] (measured by increased lectin binding). A few years later, the group of Clichy reported an increase in alpha-1,6-fucosylation in patients with acute hepatitis[29]. In contrast to the undergalactosylated glycans, these glycans are hepatocyte derived[19]. Although the pathophysiological rationale for this increased fucosylation is not fully elucidated, it is well known that N-glycan fucosylation is strictly controlled by the upregulation of specific fucosyltransferases[17]. Some data suggest that this increased fucosylation might be a marker of regeneration. In sera of HCC patients an increased abundance of branch alpha(1,3)-fucosylated triantennary glycan NA3Fb, involved in branch fucosylation, has been described[24]. The increase of serum alpha-1,3-fucosyltransferase activity is not specific for HCC but is also increased in gastric[30,31] and ovarian[32] cancer.

Furthermore, HCC patients express an increased enzymatic activity of alpha-1,6 fucosyltransferase activity[33], responsible for core-fucosylation. Recently it was shown in a partial hepatectomy model in mice that alpha-1,6 fucosyltransferase activity (Fut8) was critical for the regeneration of the liver[34]. Fut8 (-/-) deficient mice suppressed hepatocyte proliferation, which is crucial for the recovery from ALF. Interestingly, suppletion of L-fucose, which can increase GDP-fucose synthesis through a salvage pathway, significantly rescued the delayed liver regeneration of Fut8(+/-) mice. Hence, these data strongly suggest that increased fucosylation, and in particular core fucosylation, is a marker of hepatocyte regeneration during the recovery of APAP-induced ALF.

The third finding is a significant and consistent *increase of the complex* branch fucosylated *triantennary* sugar NA3Fb and the *tetraantennary sugars* NA4 and NA4Fb. Several observations support the role of increased branching in hepatocellular carcinoma, a quintessential model of regeneration. First, in Hep G2 HCC cell lines and in human serum samples of patient with HCC increased fucosylated tri-[35,36], tetra[36]-, and penta-antennary glycans were observed[37]. Second, beta1,6-GlcNac branching and thus formation of triantennary glycans is catalyzed by *N*-acetylglucosaminyltransferase 5 (GnT-V)[38,39] and is increased in human HCC samples[40]. The last argument in favor of increased branching as a marker of liver regeneration was published by Miyoshi et al.[41]. In a two-thirds partial hepatectomy model in rats, enzymatic activity of GnT-V was increased in hepatocytes and nonparenchymal cells during regeneration. Hence, the temporal changes of serum N-glycan expression reflect the complex regeneration processes associated with recovery from ALF.

In this cohort, 2 patients underwent urgent liver transplantation due to APAP-induced ALF, guided by the King's college criteria for APAP-induced ALF[8]. An assessment of the glycomic profile at admission revealed distinct glycomic profiles in both transplanted patients, that allowed to predict at admission which patients would benefit from liver transplantation. According to our data, 4 glycans, NG(1)A2F, NA2FB and NA3Fb could discriminate between both patient groups. The increased relative abundance of the NG(1)A2F isomers in patients requiring liver transplantation might be a marker of increased inflammation in the most sick patients. However, the important decrease of the relative abundance of NA3Fb in patients requiring liver transplantation might reflect the lack of sufficient regeneration capacity to recover from the ALF episode and thus the need for liver transplantation. To our knowledge, these data provide a proof-of-concept for the first biomarker that would allow to predict outcome in acute liver injury.

This study is hampered by two major shortcomings. First of all the number of patients is limited and can only provide a proof-of-concept. It needs to be confirmed in a prospective multicenter study. Second, as both transplanted patients were rapidly listed for transplantation and transplanted, we could not follow the day-by-day evolution of the glycomic profile in these patients that did not recover. It would be interesting to follow the glycomic profile in these patients beyond the day of admission.

Serum glycomic analysis was performed using capillary electrophoresis on a modified DNA sequencer, as described before[19]. The turnaround time of this analysis is less than 4 hours. During the last years, sample preparation has been simplified and it can be used on cheap high-throughput

microfluidics CE platforms e.g. the MCE-202 MultiNA, 2100 Bioanalyzer and eGene system[21], allowing an easy clinical implementation.

In conclusion, in this study we provide a sequential analysis of the whole serum glycomic profile in patients with APAP-induced ALF. Furthermore, we provide a proof-of-concept that the glycomic profile at admission is predictive for transplant-free survival in APAP-induced acute liver injury.

ACKNOWLEDGEMENTS

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VIII. General discussion and future perspectives

VIII. GENERAL DISCUSSION AND FUTURE PERSPECTIVES

This PhD research found its roots in the observation that serum proteins display typical glycoalterations in humans with chronic liver disease as opposed to healthy adults[1]. This observation is supported by the fact that the majority of circulatory proteins are secreted by the liver and thus these glycoalterations can reflect pathological processes in the liver.

Standardized protocols for sugar labeling and its application on a DNA sequencer using DSA-FACE has provided researchers with a technical environment that allows glycome analysis of the liver patient[2]. This pioneering work resulted ten years ago in the development of the first glycomics-based biomarker for the diagnosis of cirrhosis, called the GlycoCirrhoTest[3]. Although non-invasive markers for the quantification of liver fibrosis have become mainstream in clinical practice during the last 3 years[4], invasive liver biopsy was until then the standard of care for fibrosis and cirrhosis assessment.

In the introduction of this work we provided a literature review of glycomics-based biomarkers for liver disease. This overview confirmed the solid diagnostic performance of glycome changes in relation to liver disease. My research work was primarily dedicated to exploring the potential of glycomics-based biomarkers as prognostic markers. The major glycomic alterations we discovered are summarized in figure 1.

8.1.   Glycomics-based biomarkers for prognosis in liver disease

8.1.1.   Risk of HCC development in compensated cirrhosis

Liver cancer is the sixth most common cancer worldwide, the third cause of cancer-related death, and accounts for 7% of all cancers[17]. Cirrhosis is the main risk factor for the development of HCC. Although European[16,18]and American guidelines advocate ultrasonography-based screening for early HCC with or without measurement of AFP, adherence to these screening programs is disappointingly low and the incidence of HCC is rising.

In our study we were able to show that the GlycoCirrhoTest, a glycomics-based serum marker, could radically change this approach. While the current screening strategy is a one-size-fits-all approach, we showed that the use of the GlycoCirrhoTest was able to assess the risk of HCC development in patients with compensated cirrhosis and this could allow for a personalized screening protocol.

■ : N-acetylglucosamine ● : mannose ○ : galactose ▲ : fucose
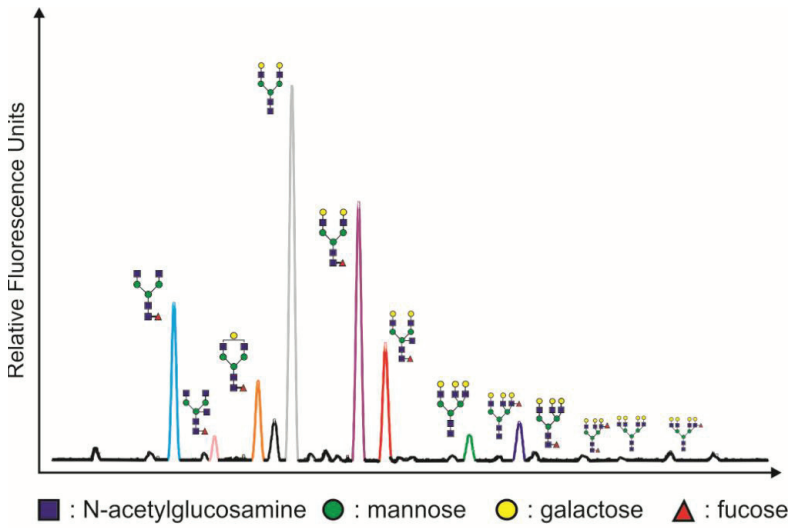
$$\text{GlycoCirrhoTest} = \text{Log} \frac{\text{(NA2FB)}}{\text{(NA3)}}$$

$$\text{GlycoTransplantTest} = \text{Log} \frac{\text{(NG1A2F)}}{\text{(NA3)}}$$

$$\text{GlycoPNFTest} = \text{NGA2F}$$

$$\text{Acute Cellular Rejection} = \frac{\text{(NA2F)}}{\text{(NA2)}}$$

$$\text{Acute Liver Failure*} = \frac{\text{(NG1A2F + NA2FB)}}{\text{(NA3Fb)}}$$
*adverse outocme

$$\text{GlycoFibroTest} = \text{Log} \frac{\text{(NGA2FB)}}{\text{(NA3)}}$$

$$\text{GlycoNASHTest} = \text{Log} \frac{\text{(NGA2F)}}{\text{(NA3)}}$$

Figure 1. Overview of glycomic alterations in relation to the disease state. Historical glycomic biomarkers GlycoFibroTest (for diagnosis of liver fibrosis) and GlycoNASHTest (for diagnosis of NASH) have also been included in this overview.

In a French cohort of 125 patients, a single measurement of the GlycoCirrhoTest, which is based on the measurement of 2 single glycans (a bisecting N-acetylglucosamine (GlcNAc)- containing N-glycan and a triantennary N-glycan) on serum glycoproteins was able to distinguish between patients with a high and a low risk of HCC development during the following 7 years. Indeed the median follow-up time in this study was 6.4 years. In a multivariate Cox Regression model including GlycoCirrhoTest, AFP and FIB-4 only GlycoCirrhoTest showed a significant and independent association with HCC development during follow up.

This finding is supported by a strong pathophysiological rationale. The enzyme GnT-III responsible for the formation of bisecting GlcNAc residues, which are the driving glycans of the GlycoCirrhoTest, is increasingly expressed in cirrhotic nodules[10,19]. It is conceivable that with more hepatocytes actively dividing in such nodules, the risk for propagation of oncogenic mutations increases and hence the risk for HCC rises. Therefore a true marker for such nodular regeneration in liver cirrhosis should also be a good risk marker for HCC, as validated here for the GlycoCirrhoTest[3].

The findings of this study could be the basis for a radical change in the approach of the cirrhotic patient. In this era of personalized medicine such tools are highly valuable. Based on the result of the GlycoCirrhoTest, patients could be stratified in a low-risk or high-risk group for HCC development. These patients could be offered personalized screening regimens. For example, this study teaches us that patients with a low GlycoCirrhoTest value have an extremely low chance of HCC development (less than 5%) in the first 3 years of follow-up, whereas this risk is higher than 40% in the patients with a GlycoCirrhoTest value. It could make sense to offer the GlycoCirrhoTest low patients a yearly screening examination, whereas the GlycoCirrhoTest High group should be followed every six months as it is performed now.

Yet, these results need to be interpreted with caution, as this is a monocentric study with a considerable but not extremely high number of patients included. However, from a statistical point of view the use of cross-validation and bootstrap validation confirmed the strong association of GlycoCirrhoTest with HCC development. This is a unique cohort with clinical data and serum that has been collected prospectively with a median follow-up time of almost 7 years. The search for similar cohorts that would allow validation has been difficult and disappointing. However, we have obtained an agreement with the French CIRRAL cohort to perform a validation of these findings in a cohort of patients with alcoholic cirrhosis and are in the phase of discussing the practicalities of this study.

A proper validation of this study does not only require an independent validation of the correlation between GlycoCirrhoTest and HCC occurrence in cirrhotic patients. We need to demonstrate that it remains valid for hard endpoints, most ideally HCC related mortality. To prove this, we would need to

follow a prospective cohort of patients and randomize them to a classic screening program with ultrasonography (with or without AFP measurement) twice yearly and to a new screening program that is adapted to the value of the GlycoCirrhoTest, where patients with a low GlycoCirrhoTest value could be offered only a yearly follow-up visit. The results of this kind of study could only be expected within 10 years.

The next step will be a pharmaco-economic evaluation of this strategy. If this strategy proves to be non-inferior to the current screening strategy, the financial repercussions could be very attractive. The reduction of visits by 50% in the GlycoCirrhoTest Low patients represents a real cost benefit, especially in countries where health insurance is not universal and where the distance to medical care can be larger than in Belgium.

In conclusion, the observation that GlycoCirrhoTest is a predictor of HCC risk in patients with compensated cirrhosis could be a highly-expected game-changer that answers the desire for personalized medicine and cost reduction in modern medicine.

8.1.2. OUTCOME AFTER LIVER TRANSPLANTATION

A major part of this PhD dissertation covers the study of the role of glycomics-based biomarkers in liver transplantation, a thusfar unexplored area. We explored the potential of glycomics in three major fields, as elaborated in chapter 4, 5 and 6. Glycome profiles were both evaluated in the perfusate of the donor liver (before transplantation) and in serum.

8.2.2.1. Glycomic analysis of perfusate

The glycomic analysis of perfusate was the most innovative and challenging field of this thesis as we did not only explore new indications for glycomic analysis but also explored the analytical and technical boundaries of our technique for glycome analysis.

The team of Callewaert et al. developed the on-membrane deglycosylation and labeling method[20] for glycan analysis in serum on a multicapillary electrophoresis-based ABI3130 sequencer. We applied this technology on perfusate samples and acquired perfusate electropherograms of excellent quality. The peak profile was comparable to the peak profile of serum showing the same peak structures.

From a theoretical point of view, perfusate analysis is an attractive alternative for liver biopsy or serum markers for the assessment of viability of the liver in liver transplantation. Perfusate is believed to represent the condition of the entire liver parenchyma and it is easy to collect in large volumes. However, only few perfusate markers have previously shown any value in the prediction of graft and patient outcome after LT[21,22].

Our study confirms the potential of perfusate analysis to guide donor allocation. In a cohort of 66 liver transplant patients, three patients developed PNF and based on the glycomic signature of perfusate *before* liver transplantation we were able to predict the occurrence of PNF *after* liver transplantation with 100% accuracy.

A few reflections should be made in the appraisal of these results.

First, this is a monocentric study with a limited number of PNF patients. Although a validation of this cohort is needed (and is on the way) to confirm these findings, we have to face that it is a clinical reality that PNF occurs in a small amount of patients, varying between 1 and 10%. Multicenter studies will be needed in order to collect enough PNF patients and we have started a multicenter trial involving all Belgian liver transplant centers to collect perfusate using a standardized protocol in order to validate these findings.

Second, the glycome signature is based on an increase of undergalactosylated glycans. The increased abundance of undergalactosylated glycans is a phenomenon that we also encountered in NASH patients and in the serum of liver transplant patients that are at greater risk of early graft loss. We know from previous reports that the undergalactosylated glycans in serum are present on IgG and not on liver-derived proteins. We measured IgG levels in perfusate and found their presence in the perfusate fluid (unpublished data). The levels of IgG were not increased in PNF patients compared to the non-PNF group. This implies that the undergalactosylation in this perfusate is a real glycomic alteration. The pathophysiological rationale is far from clear. However, it makes sense that the failing liver is a "stressed" organ, eg. by increased ischemia-reperfusion injury, with an important upregulation of inflammatory pathways, which is reflected by the increased abundance of undergalactosylated glycans. These livers are suffering and fail to develop the regeneration capacity to start functioning after liver transplantation.

Third, the accuracy for the prediction of PNF was extremely high (100%). On the other hand we found no clear relationship between the perfusate glycome profile and the occurrence of EAD. We speculate that this might 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. This information will of course not be captured in the pretransplant glycome profile. On the other hand, PNF is mainly related to very low quality grafts, which can be captured quite convincingly by the glycome profile.

If we can confirm the predictive power of perfusate glycomics for the occurrence of PNF this could have an important impact on allocation practices. Organ allocation these days is more "art" than "science". Our tools for graft assessment are limited to a clinical appreciation and the use of scores like the (ET-)DRI, which lack the prognostic power in individual patients. On the other hand, we are faced with a decreasing quality of organs due to the shift of organ donors from the "young car driver" to the older "multimorbidic stroke patient". All strategies that aim at expanding the donor pool like the use of DCD donors or the use of elderly donors have an additional negative impact on donor quality.

A reliable biomarker that can predict organ failure with 100% certainty could help us to discard these unsafe organs from the donor pool and safely use donor organs where the clinician is in doubt regarding the quality. We remind the reader that our data were obtained in real clinical practice where all organs, including the PNF patients, were considered safe by the transplant team.

Although we are convinced of the real clinical value of this glycomic marker for PNF, the current approach as published in the study reveals a major obstacle towards implementation in clinical practice. The technique that we used in the paper has a turn-around time of 48 hours because it was based on the on-membrane deglycosylation protocol[20]. In a concept where donor graft quality would be assessed before liver transplantation, this does not make any sense and makes this technique obsolete. However, we have been searching for a solution to this problem. The first logical step was the application of the newer and faster in-solution deglycosylation protocol that has been developed by the team of Prof. Callewaert[20,23] with a turn-around time of 2 to 3 hours and the potential of application on clinical CE analyzers. However, applying this protocol to perfusate was disappointing as we did not obtain reliable signals (unpublished data). The main concern is that protein concentration in perfusate is much lower than in serum. In serum the normal total protein content is between 6 and 8 g/dl, where we measured mean levels of total protein between 0.2 and 0.3 g/dl in perfusate (unpublished data). This is a dilution factor of 25 to 30. We have evaluated different techniques to concentrate the protein content of the perfusate without affecting the glycomic profile. The use of Amicon filters enabled the concentration of proteins in perfusate to a level where the in-solution deglycosylation protocol can be applied reliably. We are now in the phase of optimizing and validating this technique in order to allow rapid glycomic analysis of perfusate

(unpublished data) but have a proof-of-concept that glycomic analysis with the rapid in-solution deglycosylation protocol is feasible and reliable.

Another intriguing question is how much cold ischemia time is necessary to obtain a representative glycomic profile and how the glycomic signature changes during cold ischemia. These data are non-existing at this moment, as we have only performed measurements at the end of the cold ischemia time. An excellent experimental model to test the dynamics and value of glycomics perfusate analysis would be machine perfusion. We have approached partners to perform sequential perfusate sampling during the entire process of machine perfusion. This would allow for a systematic and comprehensive analysis of the glycomic profile and would reveal the dynamic behavior of glycomics during this process. It would also be useful to assess the potential of glycomics as biomarkers in machine perfusion.

8.2.2.2. Glycomic analysis of serum

In this project we collected and sequentially analyzed serum glycomic profiles in 127 liver transplant patients during the first year after liver transplantation. Two clinical questions were addressed. The first was whether a glycome signature during the first week after transplantation was associated with graft and patient survival during the first year after LT and the second whether a specific glycomic signature could be observed during the occurrence of ACR.

8.2.2.2.1 The GlycoTransplantTest and graft and patient survival

We defined a glycome signature that was predictive of graft and patient survival during the first year after liver transplantation. We showed that the best results were obtained by measuring the ratio of 2 glycans, an agalacto core-alpha-1,6-fucosylated biantennary glycan and a triantennary glycan as *log*(NG1A2F/NA3) on day 7 after liver transplantation. In patients with a high level of this GlycoTransplantTest, hazard ratio for graft loss was 7.222 (p<0.001; 95% CI 2.352-22.182) and hazard ratio for patient death was 3.885 (p=0.30; 95% CI 1.127-13.276) during the first year after liver transplantation.

This new biomarker opens new perspectives in transplant medicine. After liver transplantation, 7-10% of patients will require retransplantation[24]. The reasons for this retransplantation can be due to surgical/technical complications, infections, rejection, disease recurrence or other complications. The first 3 reasons prevail in the first year after liver transplantation.

Interestingly, the measurement of the GlycoTransplantTest can disclose this increased risk for graft loss only based on one measurement one week after liver transplantation. The driving glycan of the GlycoTransplantTest is an agalacto core-alpha-1,6-fucosylated biantennary glycan NG1A2F. We can presume that this undergalactosylated glycan is a reflection of the ischemia/reperfusion damage[25] and the earlier described complications that lead to graft loss, as it is well-known that these are characterized by a complex inflammatory response[26]. We speculate that the decrease of the triantennary glycan NA3 is the result of a disturbed glycosylation machinery in the failing liver and reflects the lack of regeneration capacity that is so hard needed after liver transplantation, due to a downregulation of N-acetylglucosaminyltransferase V (GnT-V).

As clinicians we are convinced that the GlycoTransplantTest is an interesting tool. Already one week after transplantation we can estimate whether the newly transplanted graft and the patient have a good chance to survive the first year after transplantation. Patients with a GlycoTransplantTest below the cut-off of as described in chapter 5 can be reassured that they have a well-functioning liver with a high chance for a good outcome. Patiënts with a high value on the GlycoTransplantTest should be monitored more carefully. In these patients for whom retransplantation is being considered, the results of the GlycoTransplantTest can support the transplant team in the decision to list the patient more rapidly for retransplantation, rather than losing unnecessary time, which will affect the patient's quality of life and might affect his general condition at the moment of retransplantation leading to a more complex surgery and recovery. To our knowledge clinical scores or biomarkers that can offer this solid prognostic value are not available.

A critical reader might suggest that our marker is a mere biomarker of EAD. We have shown however, although there is a real overlap between EAD patients and patients with a high value on the GlycoTransplantTest, that there was no significant correlation between the GlycoTransplantTest and EAD as defined by Olthoff[27]. GlycoTransplantTest showed a better prognostic value than this clinical definition with regard to graft loss and patient survival. Furthermore, in a multivariate analysis, the GlycoTransplantTest and not EAD was the only independent predictor of graft survival ($p=0.003$).

We are aware that this is a monocentric cohort study that requires validation in a second cohort. We have started collecting samples in a second cohort of patients at Ghent University Hospital from 1 January 2015 that are reaching the required follow-up time of one year soon and we plan to validate the GlycoTransplantTest in this independent cohort. Second, we have contacted other centers that are willing to provide serum samples for other historic cohorts in order to permit an external validation.

In conclusion, we believe that the GlycoTransplantTest is a powerful tool for the assessment of outcome during the first year after liver transplantation that could be used in decision-making regarding the need for liver re-transplantation. Furthermore, it could also be an interesting tool in trials studying therapeutic strategies in early graft failure where liver grafts at increased risk of graft loss could be identified.

8.2.2.2.2. Acute Cellular Rejection

The most common complication in the first year after liver transplantation is acute cellular rejection[28]. In the majority of patients ACR is easy to control and it should not affect long-term outcome if properly treated. ACR after liver transplantation is therefore not considered a major issue anymore in the transplant community. However, for a proper treatment, a proper diagnosis is needed and that is the very heart of the matter. For the diagnosis of ACR, a liver biopsy is needed. Although the risk for major complications is perfectly acceptable, a liver biopsy induces a lot of stress and anxiety to patients. Thus, a non-invasive marker for ACR would be highly appreciated.

First of all we reviewed the literature for non-invasive markers for ACR, and concluded that the results were disappointing[29]. There is no reliable and validated diagnostic biomarker for ACR.

In a cohort of 108 patients at Ghent University Hospital, ACR was observed in 38 patients. When comparing the relative abundance of every single glycan between non-ACR and ACR episodes, we discovered that 1 glycan, a core-fucosylated bi-antennary glycan NA2F was increased during ACR episodes whereas the biantennary glycan NA2 was significantly decreased during ACR episodes. However, in a general linear mixed model, the relative abundance of serum protein glycans was compared between ACR and non-ACR episodes and did not lead to a specific glycome signature that could relate to ACR.

ACR is characterized by an inflammatory infiltration of the portal tract and hepatic necrosis in severe stages of ACR[30]. Based on our insights it is not surprising that this inflammatory and damaging process in the liver affects glycosylation of liver-secreted serum proteins. The subtlety of these changes might be the reflection of the minor inflammatory process of the ACR in our cohort (more than half of ACR episodes were grade 1).

Although these results are disappointing, as we could not define a solid glycome diagnostic signature for ACR, we will continue this work. More patients are being included in this prospective cohort in order to collect more ACR patients. Furthermore we will look deeper into this matter and study intra-patient variations of glycan abundance in order to calculate deltas of the relative changes of glycans during follow up which might be more revealing of these subtle changes. In the end, if this could lead

to a reliable diagnostic marker of ACR, again it would have a major impact on clinical practice and could rival the major indication for liver biopsy after liver transplantation.

8.1.3. SERUM GLYCOMICS IN ACUTE LIVER FAILURE

ALF is a dramatic event that affects healthy and often young patients without any underlying liver disease and can require urgent liver transplantation in a minority of these patients. The need for urgent liver transplantation is based on clinical criteria, most often Kings College[31] or Clichy criteria[32] are used. In a small cohort of patients with paracetamol-induced ALF, we explored the kinetics of several glycans and searched for distinct glycomic profiles in patients who showed a spontaneous recovery of ALF and those who needed urgent liver transplantation.

The findings of this small study were exciting and intriguing. First of all we observed rapid and large changes during the occurrence and the recovery of ALF, illustrating that the serum glycans show rapid changes according to pathological processes in the liver. Second, we found a distinct glycome profile in patients that needed urgent liver transplantation compared to those who did not. We speculate that the increased abundance of the NG(1)A2F isomers might be a marker of increased inflammation in the most sick patients and that the important decrease of the relative abundance of NA3Fb in these patients might reflect the lack of sufficient regeneration capacity to recover from the ALF episode and thus the need for liver transplantation.

Again, these data need validation in larger cohorts. We have decided not to publish these data until we can refine and confirm them in more patients. Collection of patient data and serum samples is ongoing. Furthermore, we were restricted by the fact that in the two patients that needed liver transplantation, we could only collect one sample (at admission) due to the rapid liver transplantation within 24 to 48 hours. It would be interesting to study the kinetics of these culprit glycans more comprehensively in these patients.

The most widely applied prognostic system is the King's College Hospital criteria (KCH Criteria), developed from a retrospective cohort of nearly 600 patients[31]. Several studies have shown positive predictive values ranging from 70% to nearly 100% and negative predictive values ranging from 25% to 94%[33,34,35]. In a meta-analysis of studies using the KCH Criteria, the pooled sensitivity and specificity was 68% to 69% and 82% to 92%, respectively[36,37].

Although KCH Criteria have acquired an almost divine status in clinical practice, this is surprisingly not supported by the AASLD clinical practice guidelines[38] that state that *available prognostic scoring*

systems do not adequately predict outcome and determine candidacy for liver transplantation. Reliance entirely upon these guidelines is thus not recommended.

So the field is open for improvement. There is a need for robust biomarkers that can guide the clinician in the decision whether or not to transplant a patient with ALF. Although the KCH criteria have saved innumerable lives during the last decades, some patients undergo unnecessary transplantation and others are denied transplantation for no reason. I doubt that a single biomarker once will be able to appreciate the complexity of a condition like ALF. However, our preliminary data provide a proof-of-concept that the assessment of serum glycomics in ALF can identify patients in need of liver transplantation and we need to clarify the question whether glycomics can improve the prognostic accuracy of the currently used prognostic models.

8.2.   Future Perspectives

Our work in the field of glycomics as biomarkers in liver disease has given some answers to real medical questions. As it should be in research, our work has generated more uncertainty than clarity and is the source of new questions.

The first step in turning uncertainty into clarity is the validation of our new biomarkers. The most promising biomarker is the perfusate glycomarker for PNF prediction. A patent application has been filed for this marker (PCT/EP2016/065383) and we have started a multicentric validation study in the 6 Belgian liver transplant centers. Inclusion is ongoing and we plan to have a preliminary analysis by mid 2017. For these samples, the short protocol for perfusate analysis will be used. If the results of this trial confirm the proof-of-concept, industrial partners will be contacted for commercialization of this test.

Validation studies for the other markers are also in the pipeline. We have an agreement with the French CIRRAL cohort, a prospective cohort of patients with alcoholic liver cirrhosis for a validation of the GlycoCirrhoTest as predictive markers for cirrhosis. The GlycoTranplantTest will be validated in Ghent University with a new prospective cohort whose inclusion is ongoing (from 1 January 2015 onwards). Inclusion of ALF patients is also ongoing in order to increase the number of patients in this study.

Besides the validation of these glycome signatures, we plan to study the role of glycomics in new indications. First of all we will study the role of glycomics in kidney transplantation. We have hypothesized that glycomic changes, excluding the undergalactosylated glycans, are liver-specific for the reasons described before. Inclusion of kidney transplant patients is ongoing in collaboration with

Prof. Dr. Van Laecke from the department of Nephrology (Ghent University Hospital) in order to study the behavior of glycomics after kidney transplantation in comparison to liver transplantation. We expect that this exercise will increase our insights in the specific role of the liver in the serum protein glycosylation.

Another study is the role of glycomics in the prognosis of patients with HCC. Some reports have proposed that the baseline glycomic profile is predictive of the time to relapse and of the overall survival[3940]. We plan to validate these findings in our HCC-cohort.

The final project is a pan-european project supported by the European Foundation for the study of chronic liver failure (EF-CLIF)-consortium. This scientific consortium has performed cutting-edge clinical research regarding chronic liver failure and defined the concept of acute-on-chronic liver failure (ACLF) based on the CANONIC study[41]. A new prospective multicenter study, the PREDICT study, in patients with ACLF will start in 2017 and we will participate. Furthermore, an ancillary study from our center has been approved by the steering committee of the PREDICT study that will study the behavior of glycomics in ACLF. In comparison to the studies proposed in this thesis, we will try to define a glycomic signature that predicts outcome in ACLF.

8.3. CONCLUSION

In this thesis we have explored the prognostic potential of glycomics-based biomarkers in liver disease. We have identified several specific glycomic signatures in serum that are associated with a high risk of HCC development in cirrhotic patients, with graft loss and patient death after liver transplantation and with transplant-free survival in paracetamol-induced acute liver failure. Furthermore we showed that glycomic analysis of perfusate in liver transplantation is technically feasible and that pretransplant assessment of the glycome of perfusate is predictive of primary non-function after liver transplantation. All these biomarkers have a tremendous potential for translantion into clinical practice with a real impact on current clinical practice. Furthermore, all this research was based on a robust protocol for N-glycan analysis using DNA-sequencers that can easily be transferred to clinical CE-analyzers. This guarantees an easy implementation of these biomarkers in routine clinical laboratories.

Besides the potential clinical applications, this work confirms the strength of glycomic analysis for biomarker discovery. This work adds to the increasing literature that liver disease is associated with specific glycoalterations, which is supported by a strong pathophysiological rationale.

These newly discovered glycome signatures here-presented, the knowledge that serum glycoproteins are mainly liver-derived and the elegant technique for glycomic analysis are more than ever promising a sweet future for glycomics as a barometer for acute and chronic liver disease.

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IX. Summary - Samenvatting

IX. Summary

In this work we explored the potential of glycomics to detect prognostic biomarkers in liver disease and liver transplantation. Glycans are sugar structures present on proteins and lipids, and glycosylation is the most common posttranslational modification in the human body. Interestingly, most of the proteins present in serum are produced by the hepatocytes and thus, disease states that affect the liver might be reflected in an altered serum glycome profile. Our research team formerly showed the validity of this hypothesis, and based on this concept, diagnostic biomarkers were developed for the diagnosis of liver fibrosis, cirrhosis, non alcoholic steatohepatitis and hepatocellular carcinoma (HCC). The identification of these markers was possible by the development of an efficient technique applied on adapted DNA sequencers.

In this work first a literature review is provided that covers several topics : first a general introduction on chronic liver disease and liver transplantation is provided, with a focus on biomarkers for acute cellular rejection. The second part is a review of the current knowledge of glycomics-based biomarkers in liver disease.

The next sections cover original research work. First I demonstrate that the GlycoCirrhoTest, a biomarker that was formerly developed for the diagnosis of cirrhosis, is not only a diagnostic marker of cirrhosis, but also a prognostic marker that can predict whether a patient with compensated cirrhosis has a low or a high risk to develop HCC. This is a new tool that could be used in the follow-up of patients with cirrhosis, as these patients need six-monthly screening for HCC by ultrasonography. This GlycoCirrhoTest could help in the development of personalized screening protocols for cirrhotic patients according to the value of this test.

In a next section we assessed the potential value of glycomics to develop prognostic biomarkers in the field of liver transplantation. We first showed that it was technically feasible to measure glycome profiles on perfusate, the fluid in which the liver is transported from the organ donor to the liver recipient. We successfully identified a specific glycome signature that is 100% predictive of a patient's risk for developing primary non-function, a dramatic complication in the first hours upon liver transplantation that requires urgent retransplantation. This biomarker could prevent the transplantation of unsafe donor livers. We also studied serum glycome profiles after liver transplantation and discovered that a specific glycome signature 1 week after liver transplantation serves as an independent predictor of graft loss during the first year after liver transplantation. This glycome marker could be an additional tool to guide transplant physicians in the decision to select patients for retransplantation when a liver graft shows suboptimal liver function.

In chapter 6 we searched for a specific glycome signature that could guide clinicians in the diagnosis of acute cellular rejection (ACR), which is the most important indication for liver biopsy after liver transplantation. Although, we found significant glycome alterations during the development of ACR, these findings cannot currently be used as a diagnostic marker for ACR.

Finally we studied glycome alterations during the development and recovery of paracetamol-induced acute liver failure. We were able to define a specific glycome signature that was congruent with the Kings College criteria for the selection of patients who were in need of urgent liver transplantation.

In conclusion, the work described in this PhD-dissertation adds to the increasing evidence that the whole serum protein glycome is a robust surrogate marker of processes that affect the liver. Furthermore, we were able to identify several potential prognostic biomarkers with real clinical utility in cirrhosis and liver transplantation. Validation studies of the most promising glycomics-based biomarkers are ongoing.

SAMENVATTING

In dit werk hebben we het potentieel verkend van "glycomics" als prognostische biomerkers in leverziekten en levertransplantatie. Glycanen zijn suikerstructuren die aanwezig zijn op eiwitten en vetten, en glycosylatie is de meest voorkomende post-translationele modificatie in het menselijk lichaam. De meeste eiwitten die in het serum van mensen aanwezig zijn worden geproduceerd door de levercellen. Ziektetoestanden die de lever aantasten zouden dus kunnen vertaald worden in een gewijzigd glycosilatieprofiel in het serum.

Ons onderzoeksteam heeft vroeger al aangetoond dat deze hypothese correct is. Gebaseerd op dit concept werden de laatste jaren diagnostische biomerkers ontwikkeld die de diagnose kunnen bevestigen van leverfibrose, levercirrose, niet alcoholische steatohepatitis en primaire leverkanker of hepatocellulair carcinoma (HCC). Dit was mogelijk dankzij de ontwikkeling van een performante techniek die toegepast wordt op DNA sequencers.

Deze doctoraatsthesis bestaat vooreerst uit een literatuuroverzicht dat diverse onderwerpen overschouwt. Eerst wordt een algemene inleiding over chronische leverziekten en levertransplantatie gegeven, met een focus op biomerkers voor acute cellulaire rejectie (of afstoting van de lever). Het tweede deel van de inleiding geeft een overzicht van de huidige stand van zaken aangaande diagnostische merkers voor leverziekten gebaseerd op glycomics-technologie.

De volgende hoofdstukken bevatten origineel werk. Ten eerste hebben we aangetoond dat de GlycoCirrhoTest, een biomerker die voorheen ontwikkeld werd voor de diagnose van levercirrose, niet alleen een diagnostische merker is, maar ook een prognostische merker die kan voorspellen of patiënten met levercirrose een groot of een laag risico hebben om de daaropvolgende jaren een hepatocellulair carcinoma te ontwikkelen. Op dit ogenblik worden patiënten met levercirrose om de 6 maanden opgevolgd met een echografie gezien het hogere risico op HCC. Met deze biomerker zouden we patiënten een gepersonaliseerd opvolgingsprotocol kunnen aanbieden naargelang hun risicoprofiel op HCC.

In een volgend hoofdstuk werd het potentieel van glycomics in het domein van levertransplantatie onderzocht. Eerst hebben we aangetoond dat het technisch mogelijk is om glycomics te bepalen op perfusievloeistof, de vloeistof waarin de donorlever vervoerd wordt van de donor naar de patiënt die de levertransplantatie ondergaat. We konden met succes een specifiek glycosylatieprofiel bepalen dat met 100% nauwkeurigheid kon aantonen welke patiënten na de transplantatie een *primary non function* ontwikkelden, een ernstige complicatie waarbij de nieuwe lever niet goed functioneert en waarbij een dringende hertransplantatie noodzakelijk is. Het gebruik van deze biomerker zou kunnen toelaten om onveilige donororganen niet te gebruiken voor levertransplantatie.

Daarnaast hebben we ook aangetoond dat de analyse van het glycosylatieprofiel in serum op dag 7 na levertransplantatie een onafhankelijke voorspeller van de kans op verlies van het de donorlever binnen 1 jaar na levertransplantatie. Deze biomerker zou een nuttig hulpmiddel kunnen zijn voor transplantatieartsen in de beslissing om een patiënt met een lever die slecht functioneert na levertransplantatie opnieuw te transplanteren.

In hoofdstuk 6 onderzochten we of we een specifiek glycosylatieprofiel in serum konden vinden voor de diagnose van acute cellulair rejectie of afstoting. We vonden significante veranderingen in het glycosylatieprofiel tijdens afstoting van de transplantlever, maar de wijzigingen waren onvoldoende om nu als biomerker gebruikt te worden.

Tenslotte hebben we glycosylatieveranderingen bestudeerd bij patiënten die een acuut leverfalen ontwikkelen ten gevolge van paracetamol. We konden hierbij een specifiek glycosylatieprofiel identificeren dat voorspellend is voor de noodzaak aan een dringende levertransplantatie, congruent met de zogenaamde Kings College criteria.

We kunnen dus concluderen dat het werk dat in deze doctoraatsthesis is voorgesteld extra bewijs brengt dat de glycoomanalyse van bloed op robuuste wijze reflecteert wat zich in de lever afspeelt. Bovendien konden we verschillende potentiële biomerkers ontwikkelen met een reële klinisch nut bij patiënten met levercirrose en levertransplantatie. Validatiestudies zijn lopende voor de meest veelbelovende onder deze glycosylatiemerkers.

X.   Curriculum Vitae

PERSONAL INFORMATION

Xavier Patrick Danielle Marie Jozef **VERHELST**

Born 26 April 1981 in Gent (Belgium)

Belgian Nationality

Married to Sofie Taverniers, father of Henri (°2017)

Department of Hepatology and Gastroenterology

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De Pintelaan 185

B-9000 Gent (Belgium)

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ACTUAL POSITION

2012-present **Gastroenterologist/Hepatologist**

From 2012 staff member ("Resident") and since 2016 consultant-hepatologist ("Adjunct-Kliniekhoofd") at the department of Hepatology and Gastroenterology (Ghent University Hospital, Ghent, Belgium) with focus on Hepatology and Liver Transplantation.

2013-2015 **Clinical Ph.D. Fellowship** (parttime) from **the Research Foundation Flanders** (Fonds voor Wetenschappelijk Onderzoek – FWO) for the project "Glycomics and Liver Transplantation".

EDUCATION

1999-2006 **University/Medical School**

Faculty of Medicine and Health Sciences, Ghent University (Belgium)

Master of Medicine (MD) - Diploma "Arts" - *Magna Cum Laude* (2006)

1993-1999 **Secondary School**

Sint-Barbaracollege, Gent, Belgium (Greek-Latin)

POSTGRADUATE TRAINING

2006-2012   Resident in Internal Medicine (Ghent University Hospital – Dpt. of Internal Medicine)

Resident in Imeldaziekenhuis (Bonheiden), AZ Maria Middelares (Gent), and Ghent University Hospital

2009-2012   Resident in Gastroenterology and Hepatology

Resident in AZ St Jan Brugge (Bruges) and Ghent University Hospital

Specialist in **Gastroenterology and Hepatology** (Belgian Board Certification) since 1 October 2012

FELLOWSHIP

2013-2014   **Hôpital Beaujon, Clichy, France**. Prof. Dr. François Durand

Visiting fellow (4 months) in the department of Hepatology/Liver Transplant Unit

Laureate of a "Horlait Dapsens" Bursary

ADDITIONAL TRAINING (RELEVANT SELECTION)

2016-present   Abbvie Hepatology Academy (2016-2019)

Three-year course including yearly stand alone meeting and 6 preceptorships in European

centers of excellence in Hepatology

2016 : Preceptorship King's College, London (UK) and University Hospital Bialystok (Poland)

2015   Preceptorship Liver Transplantation and HCV

June 2015 Centre Hepatobiliaire, Paul Brousse, Paris (France)

2015   Statistical analysis using SPSS, Center for Biostatistics, Ghent Univerisity

Basic (2013) and Advanced Course (2015)

2013   Certificate in Transplantation - European Society for Organ Transplantation (ESOT)

Due to participation and passing examination of the Introductory program for

Transplantation (IPT) and Hesperis Course, organized by the European Society for

Organ Transplantation (ESOT)

May 2013, Sofia Antipolis (France)

2013   Introductory course to Glycobiology, Ghent University, Faculty of Sciences

2012   European Transplant Fellow Workshop (ETFW) organized by ESOT,

Vienna (Austria), October 2012

2012   Presentation Skills Workshop

Janssen Academy, Rome (Italy), May 2012

2012   Principles of ICH Good Clinical Practice Training Course

                Boehringer Ingelheim, Brussels (Belgium) October 2012

                Update course in November 2015, Ghent University Hospital (Belgium)

INTERNATIONAL EXPERIENCE

2013-2014   Hôpital Beaujon, Clichy, France

                Fellowship Hepatology and Liver Transplantation in Hôpital Beaujon (Clichy, France),

                Prof. Dr. F. Durand (4 months)

2006   Saint Anthony's Hospital, Tororo, Uganda

                Voluntary co-worker, supported by a BIOS travel grant (2 months)

2004   Centre Hospitalier Universitaire Vaudois (CHUV), Lausanne, Switzerland

                Internship during 2 months in the Department of Internal Medecine, hosted by an Erasmus exchange

                program (Prof. Dr. P. Nicod)

                Internship during 1 month in the Department of Cardiovascular Surgery (Prof. Dr. L. K. Von Segesser)

2002   Oruro, Bolivia

                Internship in a medical centre in a deprived area, supported by the non-governmental organization

                CIMIC VZW

2001   Sectia Psihiatrie, Gheja, Romania

                Voluntary coworker in a psychiatric clinic organized by the non-governmental association RoeMeenJe

                VZW

SCIENTIFIC WORK

Member of International Study Groups

Global Primary Biliary Cholangitis (PBC) research group

Vascular Liver Disease Interest Group (VALDIG)

Publications in Journals of the Science Citation Index

De Rycke L, **Verhelst X**, Kruithof E, Van den Bosch F, Hoffman IEA, Veys EM, De Keyser F: Rheumatoid Factor, but not Anti-Citrullinated Protein Antibodies, is Modulated by infliximab Treatment in Rheumatoïd Arthritis. Ann Rheum Dis 2005; 64: 299-302 (IF : 8.7)

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Verhelst X, Cabooter M, De Wilde V, Laukens P, Orlent H. A case of emphysematous gastritis. Endoscopy 2012; 44 Suppl 2:E159 (IF : 5.2)

Verhelst X, Troisi R, Colle I, Geerts A, Van Vlierberghe H. Biomarkers for the Diagnosis of Acute Cellular Rejection in Liver Transplant Recipients : A Review. Hepatol Res. 2013 Feb;43(2):165-78. (IF : 2.2)

Raevens S, De Pauw M, Reyntjens K, Geerts A, **Verhelst X**, Troisi R, Berrevoet F, Rogiers X, Van Vlierberghe H, Colle I. Oral vasodilator therapy in patients with moderate to severe portopulmonary hypertension as bridge to liver transplantation. Eur J Gastroenterol Hepatol. 2013 Apr;25(4):495-502. (IF : 1.757)

Colle I, **Verhelst X**, Vanlander A, Geerts A, Van Vlierberghe H, Berrevoet F, Rogiers X, Troisi R. Pathophysiology and management of post resection liver failure. Acta Chir Belg 2013, 113 (3), 155-161 (IF : 0.43)

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Coussens E, Rogge S, Haspeslagh M, Geerts A, **Verhelst X**, Van Vlierberghe H, Troisi RI, Colle I. Cutaneous infection by Alternaria infectoria in a liver transplant recipient: a case report. Acta Gastroenterol Belg, 2014 Jun;77(2):256-8. (IF : 0.638)

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Vandenabeele L, **Verhelst X**, Pieters A, Van Vlierberghe H, Geerts A. Endoscopische bandligatie : nieuwe therapie voor therapieresistente "gastric antral vascular ectasia" of watermelonemaag? Tijdschrift voor Geneeskunde 2015;71:1312-1315.

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Janssens M, **Verhelst X**, Hindryckx P, De Looze D, De Vos M, Peeters H. Double balloon enteroscopy is an adequate therapeutic tool to address angiodysplasia-related anemia. XXVth Belgian Week of Gastroenterology 2013, Antwerp. Acta Gastroenterologica Belgica 2013; 76: G03

De Man K, Defreyne L, Delanghe E, Smeets P, **Verhelst X**, Geerts A, Van Vlierberghe H, Rogiers X, Troisi R, Lambert B. Treatment of hepatocellular carcinoma with intra-arterial Yttrium-90 microspheres for downstaging patients to transplantation. European Association of Nuclear Medicine, EANM, Lyon, October 2013, OP694; EJNMMI 2013;40(2):S248-S248.

Verhelst X, Vanderschaeghe D, Castera L, Geerts A, Goutté N, Francoz C, Durand F, Callewaert N, Van Vlierberghe H. Assesment of serum glycomics (GlycoCirrhoTest) for risk prediction of hepatocellular carcinoma development in patients with cirrhosis. Oncopoint UGent, February 2015, AWARD BEST PRESENTATION

Vandewynckel Y, Laukens D, Devisscher L, Paridaens A, **Verhelst X**, Van den Bussche A, Raevens S, Van Steenkiste C, Van Troys M, Ampe C, Descamps B, Vanhove C, Govaere O, Libbrecht L, Geerts A, Van Vlierberghe H. Tauroursodeoxycholic acid dampens oncogenic apoptosis induced by endoplasmic reticulum stress during hepatocarcinogenic exposure. Oncopoint UGent, February 2015 (Bullet Presentation)

Verhelst X, Vanderschaeghe D, Castera L, Geerts A, Goutté N, Francoz C, Durand F, Callewaert N, Van Vlierberghe H. Assesment of serum glycomics (GlycoCirrhoTest) for risk prediction of hepatocellular carcinoma development in patients with cirrhosis. Belgian week of Gastroenterology, February 2015, Brussels. Acta Gastroenterologica Belgica 2015; 78;1: Abstract A25.

Degré D, Laleman W, **Verhelst X**, Lamproye A, Vanwolleghem T, Gustot T, Starkel P, Lanthier N, Michielsen P, Delwaide J, Vanvlierberghe H, Nevens F. Multicenter Belgian experience of Sofosbuvir (Medical Need Program) in very difficult-to-treat HCV patients: safety and efficacy results. Belgian week of Gastroenterology, February 2015, Brussels, Acta Gastroenterologica Belgica 2015; 78;1.

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HONOURS

2012: Laureate of the Horlait Dapsens foundation for the project Glycomics and Liver transplantation

2012: Belgian Week of Gastroenterology Research Grant

2013: ESOT Short Stay and Study Grant.

2015: Oncopoint Ghent Unversity. Award for Best Presentation

2015: EASL Vienna : Young Investigator Travel Grant

2015: UEG Barcelona: Young Investigator Travel Grant

2017: Belgian Transplant Society Meeting: Award for Best abstract

CLINICAL TRIALS

Principal investigator or subinvestigator in more than 30 clinical phase I,II and III trials in drugs for hepatitis C, HCC, NASH, acute alcoholic hepatitis amongst others.

REVIEWER

Journal of Hepatology, Oncotarget, World Journal of Hepatology, World Journal of Gastroenterology, Acta Gastroenterologica Belgica and Acta Clinica Belgica

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Board member of Flemish Society of Gastroenterology (Vlaamse Vereniging voor Gastroenterologie)

Belgian Liver and Intestine Transplantation Committee (Substitute)

Belgian Society for the Study of the Liver (BASL), European Society for Organ Transplantation (ESOT), European Association for the Study of the Liver (EASL), Belgian Transplantation Society (BTS)

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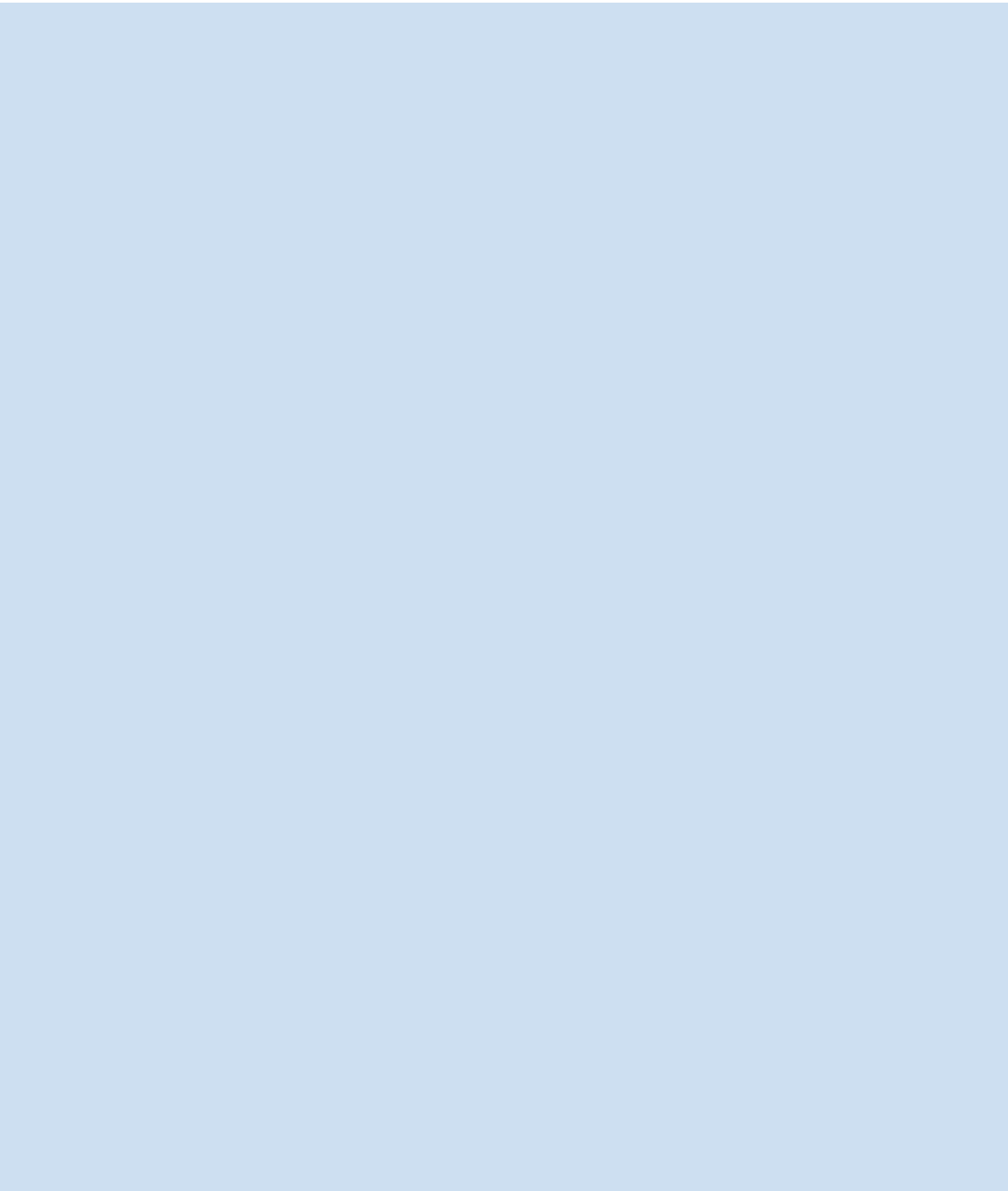
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Xavier

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