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

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Statement of Translational Relevance

This prospective study demonstrates that a simple serum blood test based on the analysis of serum glycomics can discern patients with compensated cirrhosis with a high risk for development of hepatocellular carcinoma (HCC) from patients with a low HCC risk. Screening strategies for patients with compensated cirrhosis could be guided by a positive test on GlycoCirrhoTest. This is the first biomarker that allows for a powerful risk stratification in this population. Interestingly, this biomarker is supported by a strong pathophysiological rationale. Furthermore, this technique for glycomic assessment can be easily implemented on routine capillary electrophoresis-based analysers, widely available in routine hospital laboratory facilities.

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 3.37-31.43; 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 1.58-13.60).

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.
Introduction

Hepatocellular carcinoma (HCC) represents up to 85% of the primary liver cancer burden (1). In recent years, an increasing number of biomarkers have been proposed for the diagnosis of HCC (2). Other markers have been proposed to better assess the prognosis of the outcome of HCC (3). 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 most prevalent aetiologies of chronic liver disease, the vast majority of HCC cases originate on a background of cirrhosis (4)(5)(6)(7), 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% (8). 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 (9)(10). The aim of screening is to detect small tumours with more chance of curative therapy (11). This screening strategy in cirrhotic patients showed a reduction in HCC mortality rates (12) and it is cost-effective (13). 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 (14). 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\(^\text{15,16}\), 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 $\beta1,4$ linkage to the $\beta$-linked mannose of the trimannosyl core structure of N-linked oligosaccharides of glycoproteins, using UDP-GlcNAc as donor substrate\(^\text{17}\). 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\(^\text{18,19}\). 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\(^\text{20,21}\). After treatment of HCC with transarterial chemoembolization or percutaneous ablation, serum GnT-III levels decreased markedly\(^\text{20,21}\). 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\(^\text{22}\) and alpha foetoprotein (AFP).
Patients and methods

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

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(24) 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).
**Design**

A glycomic fingerprint including the GlycoCirrhoTest was obtained on serum samples collected at enrolment in this prospective cohort study(23) 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(24). When imaging was not conclusive, patients had a US guided-biopsy. Patient characteristics and routine laboratory values at inclusion are summarised in Table 1.

**Glycomic analysis**

Five microliter of serum were processed according to the in-solution deglycosylation method described by Vanderschaeghe et al.(15). 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(25). 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(16). 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)(26).

**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).
**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.

**Results**

**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
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.

**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(26). 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: 3.372-31.426, 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.

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
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.

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).

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.

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).

Total baseline glycomic fingerprint and risk for developing HCC

The blood serum N-glycan electropherogram yields 13 glycans that have been identified before (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).

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.

\[
\text{GlycoHCCRiskScore} = [(\text{NGA2FB} \times 0.137) + (\text{NA2FB} \times -0.044) + (\text{NA3} \times -0.216) + (\text{NA3F} \times 0.158) + (\text{NA3Fbc} \times 0.796) + (\text{NA4} \times -0.764)].
\]

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.
Discussion

Although many biomarkers have recently been developed for the diagnosis of (early) HCC, 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(26) 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(18,19). Furthermore, GnT-III activity is gradually increased in sera and nodular liver tissue of cirrhotic patients without and with HCC(21)(20). Importantly, we previously showed that GlycoCirrhoTest is specifically increased in cirrhotic patients, but not in patients with earlier stages of liver fibrosis(26), 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(26).
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 performed 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(9)(10) 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.

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References


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

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

<table>
<thead>
<tr>
<th>Number of HCC cases</th>
<th>GlycoHCCRiskScore</th>
<th>GlycoCirrhoTest</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCC within 2 years</td>
<td>3</td>
<td>0.605 (0.274-0.896)</td>
</tr>
<tr>
<td>HCC within 3 years</td>
<td>6</td>
<td>0.703 (0.401-0.896)</td>
</tr>
<tr>
<td>HCC within 4 years</td>
<td>10</td>
<td>0.798 (0.633-0.901)</td>
</tr>
<tr>
<td>HCC within 5 years</td>
<td>14</td>
<td>0.803 (0.566-0.899)</td>
</tr>
<tr>
<td>HCC within 6 years</td>
<td>17</td>
<td>0.771 (0.610-0.881)</td>
</tr>
<tr>
<td>HCC within 10 years</td>
<td>34</td>
<td>0.729 (0.602-0.856)</td>
</tr>
</tbody>
</table>
**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 agalacto, core-alpha-1,6-fucosylated biantennary structures (NG1A2F), peak 5 is the bigalacto biantennary glycan NA2, peak 6 is the bigalacto, core-alpha-1,6-fucosylated biantennary glycan NA2F, peak 7 is the bigalacto, core-alpha-1,6-fucosylated bisecting biantennary glycan NA2FB, peak 8 is the triantennary glycan NA3, peak 9 is the branching alpha-1,3-fucosylated triantennary glycan NA3Fb, peak 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-acetylgalactosamine 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-acetylgalactosamine (GlcNAc) residue from the uridine diphosphate (UDP)-GlcNAc donor to core-mannose in a β 1-4 configuration and forms bisecting GlcNAc.
Figure 2: Flowchart with overview of inclusion- and exclusion criteria.
**Figure 3. 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)).
**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.
Figure 1

A

Relative Fluorescence Units

NGA2F
NG(1)A2F
NA2
NA2F
NA3Fb
NA3Fbc
NA3Fc
NA4
NA4Fb
NA2FB

Time

B

Relative Fluorescence Units

Low GCT
NA2FB
NA3

High GCT

Time

C

UDP-GlcNAc
GnT-III

UDP

NA2FB

: N-acetylglucosamine
: mannose
: galactose
: fucose
133 patients

Inclusion criteria

- Biopsy Metavir F4
- CHILD Pugh A or B
- Absence of HCC
- Serum sample between 1995 and 2005

125 patients

Exclusion criteria

- HCC within 1 year (n=6)
- FU < 1 year (n=2)