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Input of serum haptoglobin fucosylation profile in the diagnosis of hepatocellular carcinoma in patients with non-cirrhotic liver disease.

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Abstract

Background: Haptoglobin bifucosylated tetra-antennary glycan have been identified in patients with early stage hepatocellular carcinoma, but its specificity according to the presence or not of cirrhosis has never been assessed. The aims of this study were to determine if haptoglobin bifucosylated tetra-antennary glycan 1) could be a marker of HCC in patients without cirrhosis 2) could increase the performance of standard alpha-fetoprotein (AFP) or recent blood tests for HCC detection, *i.e.*, lectin-reactive alpha-fetoprotein (AFP-L3), des-gamma-carboxy prothrombin (DCP) and Liver-Cancer-Risk-test (LCR1-test).

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Author contribution to manuscript:

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Conflict of interest statement

Thierry Poynard is the inventor of FibroTest, SteatoTest, SteatoTest 2, NashTest, NashTest 2, LCR1 and LCR2 tests, founder of BioPredictive, the patents belong to the public organization Assistance Publique-Hôpitaux de Paris (AP-HP).

Valentina Peta and Yen Ngo are full employees of BioPredictive.

The other authors have nothing to declare: Chantal Housset, David M. Lubman, Francoise Imbert Bismut, Gérard Bolbach, Gilles Clodic, Jianhui Zhu, Keyvan Rezai, Lucrèce Matheron, Raluca Pais, Samuel Huguet.

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Methods: We retrospectively selected patients, 102 with HCC (21 without cirrhosis), matched by stages with 140 controls without HCC (81 without cirrhosis). Haptoglobin fucosylation was assessed by MALDI-TOF. LCR-glycan algorithm was constructed combining components of the LCR-1 test (haptoglobin, gammaglutamyl-transpeptidase, apolipoproteinA1, alpha-2-macroglobulin) with AFP, AFP-L3, DCP and haptoglobin bifucosylated tetra-antennary glycan.

Results: In 102 patients without cirrhosis (21 HCC and 81 controls), the intention-to-diagnose analyses showed that haptoglobin bifucosylated tetra-antennary glycan alone had a sensitivity of 71% (15/21;95%CI 50–86), significantly better ($P=0.02$) than standard AFP (43%;9/21;95%CI 24–63), and a specificity of 96% (78/81;95% 90–99). The sensitivity of LCR-glycan, in patients without cirrhosis, was 86% (18/21; 95%CI 63–95) significantly better ($P=0.001$) than standard AFP (43%; 9/21; 95%CI 24–63), with an AUROC of 0.943 (95%CI 0.806–0.98) compared to 0.811 (95%CI 0.630–0.908) for AFP ($P=0.06$).

Conclusion: Haptoglobin bifucosylated tetra-antennary glycan is associated with the presence of HCC in patients with chronic liver disease including those without cirrhosis. Its combination with existing HCC biomarkers could improve the performance of standard AFP for HCC detection.

Keywords

Haptoglobin; fucosylation; hepatocellular carcinoma; glycan; cirrhosis

Introduction

Hepatocellular carcinoma (HCC) is the fourth most common cancer worldwide and the third leading cause of cancer-related mortality. It mainly develops in patients with chronic liver disease [1]. Patients who are diagnosed with early stage HCC have a 5-year survival rate of nearly 70%, while 2-year survival decreases significantly to 15% in patients diagnosed at later stages [2]. Thus, the development of an early non-invasive method for the detection and prediction of HCC in patients with chronic liver disease, with or without cirrhosis is highly important.

The most common existing non-invasive detection of HCC relies on serum markers and imaging with abdominal ultrasound every 6 months in high-risk patients, such as those with cirrhosis [3]. The European Association for the Study of Liver disease (EASL) guidelines recommend to include all patients at high risk of developing HCC, such as cirrhotic patients, into surveillance programs. Surveillance include abdominal ultrasound every six months. Non-cirrhotic F3 patients, regardless of etiology may be considered for surveillance based on an individual risk assessment [4].

Alpha-fetoprotein (AFP) has been used as a tumor marker of HCC since the 1970's. However, the sensitivity of AFP is low, with a high false negative rate for the detection of early stage cancer [5]. Moreover, this marker is often mildly elevated in patients with chronic hepatitis or cirrhosis in the absence of HCC [6,7]. Thus, while AFP is an option for the surveillance of patients with cirrhosis in 2018 AASLD guidelines [8], it is not recommended in EASL guidelines [4].

Des-gamma-carboxy prothrombin (DCP), an abnormal prothrombin protein that is found at higher levels in the serum of HCC patients, has been used as an alternative marker for the diagnosis of HCC [9]. However, the diagnostic value of DCP alone varies depending on patient characteristics [10], and current guidelines only recognize its performance for the stratification of the risk of HCC but do not recommend its use in the surveillance of patients with cirrhosis.

In the past few years different fucosylated proteins have been reported as potential cancer biomarkers [11–13]. For example, fucosylated alpha-fetoprotein (AFP-L3), a component of AFP with a core fucose residue, has been considered as a potential biomarker for HCC [14].

Serum haptoglobin (Hp), an acute-phase liver protein, has attracted particular attention as a target for aberrant glycosylation in liver disease [15]. Recently, high levels of bifucosylated tetra-antennary haptoglobin glycan (BiFc-tetra-glycan), with both core and antennary fucosylation have been identified in patients with early stage HCC compared to patients with cirrhosis [16–18]. These results suggest that bifucosylated tetra-antennary glycan may be a potential marker for the early detection of HCC. However, these studies have several limitations. In particular, HCC patients were not stratified into those with and without cirrhosis, making it impossible to determine if the altered expression of this glycan was only correlated to a greater prevalence of cirrhosis in these subjects. Moreover, the specificity of bifucosylated tetra-antennary glycan was not assessed according to the severity of liver fibrosis.

We recently constructed two multi-analyte blood of HCC risk, LCR1 and LCR2, with good performances in patients with chronic liver disease [19]. LCR1 (Live Cancer Risk 1) is a multi-analyte blood test for the early stratification of the risk of cancer, able to identify patients without cirrhosis with a high risk of liver cancer at 10 years. LCR1 combines two proteins associated with liver tissue repair, apolipoprotein A1 (ApoA1) and haptoglobin (Hp) [20,21], three factors previously shown to be associated with HCC risk, gender, age and gammaglutamyl-transpeptidase (GGT) [22] and adjustment for a fibrosis biomarker, alpha2-macroglobulin. LCR2 (Live Cancer Risk 2), combining the components of LCR1 test with AFP, showed better performance than AFP alone for the prediction of the occurrence of cancer at 5 years, both in patients without and with cirrhosis..

Our results showed that assessing LCR1 in patients without cirrhosis, and LCR2 both in patients with cirrhosis or in those without cirrhosis but with high LCR1, should improve the efficiency of the AASLD-standard surveillance, which currently includes ultrasonography, with or without AFP limited to patients with cirrhosis [8,19].

The primary aim of this proof of concept study was to assess if bifucosylated tetra-antennary glycan could be a marker of hepatocellular carcinoma in patients with non-cirrhotic liver disease.

The second aim was to construct a potential new liver-cancer multi-analyte blood test LCR-glycan (patent pending). We assess if this test could increase the performance of standard markers (AFP, AFP-L3, DCP) or recent blood tests (LCR-1) for the very early detection of HCC risk in non-cirrhotic patients.

Patients and Methods:

Serum samples and study

We retrospectively selected patients with available frozen serum stored at -80°C from 2012 to 2015, without previous HCC or liver transplantation. Patients belong to the “Groupe Hospitalier Pitié-Salpêtrière” prospective cohort of FibroFrance, a program that began in 1997 (Clinical registry number: NCT01927133). The protocol was approved by the institutional review board, appropriate regulatory agency and performed in accordance with principles of Good Clinical Practice. All patients provided written informed consent before entry.

Characteristics of patients included in the retrospective LCR-glycan subset compared to the all the patients of the FibroFrance cohort are showed in Supplementary Table S1. The study included 242 patients, 102 with recent HCC (21 with cirrhosis et 81 without cirrhosis) and 140 controls without HCC (59 with cirrhosis and 81 without cirrhosis) (Figure 1). The different seven stages of liver fibrosis/cirrhosis, including the 4 non-cirrhotic stages (F0, F1, F2, F3) and the 3 critical steps in cirrhosis (F4.1, F4.2, F4.3) were assessed according to predetermined FibroTest cutoffs. F4.1 was defined as cirrhosis without varices or severe events, F4.2 was defined as the presence of varices without severe events and F4.3 was defined as the occurrence of severe event such as variceal hemorrhage and hepatic insufficiency [23].

Most published HCC risk scores have included histological cirrhosis as a major component, which is a limitation due to the adverse events and the cost of biopsy. The FibroTest is a validated fibrosis biomarker which can replace a biopsy to determine the presence or absence of cirrhosis when constructing new tests [23,24].

The diagnosis of HCC was based on non-invasive imaging, including multiphasic computed tomography (CT) and magnetic resonance imaging (MRI), and on histological examination by an experienced pathologist [7,8]. All HCC patients were classified according to Milan criteria staging system [25].

Isolation of haptoglobin from serum

Haptoglobin was purified from 25 μL of serum using an anti-haptoglobin antibody immobilized column [26]. The purification was performed on an Alliance high performance liquid chromatography system (Waters, Milford, MA, USA) with a flow rate of 0.5 mL/min for 40 min. The haptoglobin bound fraction was eluted with a stripping buffer (0.1 mol/L glycine, pH 2.5) and neutralized with a neutralization buffer (0.1 mol/L Tris-HCl, pH 8.0). The eluted fraction (~ 3 mL) was desalted using a 4 mL YM-3 centrifugal device (Sigma-Aldrich) and then dried in a SpeedVac concentrator.

Deglycosylation and desialylation of haptoglobin

To simplify the glycan spectrum and improve the sensitivity of the method, haptoglobin desialylation was performed. The eluted haptoglobin was denatured by adding 1 μL of denaturing buffer (0.2% sodium dodecyl sulfate, 100 mmol/L β -mercaptoethanol) and

incubation at 65°C for 30 min. One unit of peptide N-glycosidase F (PNGase F) (New England Biolabs) was added to release N-glycan. The reaction mixture was incubated at 37°C overnight and quenched by heating at 95°C for 10 min. The mixture was dried, followed by desialylation with 40 mU of neuraminidase (Sigma-Aldrich) at 37°C overnight. The desialylated N-glycan were dried, redissolved in 10 µL of water (with 0.1% trifluoroacetic acid), and then purified using porous graphitized carbon tips [27].

Permethylation of glycan and MALDI-TOF analysis

The glycans were permethylated according to an established procedure [28]. One microliter of the matrix solution, alpha-cyano-4-hydroxycinnamic acid (HCCA) (Sigma-Aldrich), 12 mg/mL in acetonitrile/water/TFA 1/1/0.1%, was spotted on the MALDI plate and allowed to air dry, then 1 µL of the sample solution was deposited on the dried matrix layer. Glycan were analyzed in positive ions reflector mode on a MALDI TOF-TOF (4700 Proteomics Analyzer, Applied Biosystems). When total serum haptoglobin was 0.1g/L enabling the identification of N-glycan, the assay was defined as not applicable. Serum haptoglobin levels were below the limit of detection in 24 HCC patients and 20 controls.

Measurement of AFP, AFP-L3 and DCP

AFP and AFP-L3% were measured in the same serum by microchip capillary electrophoresis and liquid-phase binding assay on a µTAS Wako i30 auto analyzer (FUJIFILM Wako Chemicals, Neuss, Germany). The measuring range for AFP was 0.3–1000 ng/mL. The percentage of AFP-L3 can be measured when serum concentrations of AFP are above 0.3 ng / mL. DCP was measured by chemiluminescence enzyme immunoassay on a Lumipulse® G120 analyzer (Fujirebio, Belgium). DCP serum concentrations were expressed as mAU/mL. The standard cutoffs were used for defining positive results, >150 mAU/ml (7.5 ng/mL) for DCP, and ≥ 10% for AFPL-3 and ≥20 ng/ml for AFP [29].

Biochemical analyses

The FibroTest component assays were performed on an automatic analyzer Modular P from Roche Diagnostics (Mannheim, Germany). Proteins concentrations were measured according to turbidimetric analytical methods using manufacturer's reagents for haptoglobin and ApoA1, and Diagam (Ghislenghien, Belgium) reagents for alpha-2-macroglobulin. GGT was determined using Szasz method and calibrator value given for the international federation of clinical chemistry (IFCC).

Construction of LCR-glycan algorithm and statistical analysis

The algorithm (LCR-glycan) was constructed by logistic regression by combining total haptoglobin, bifucosylated tetra-antennary haptoglobin glycan, GGT, apoA1, alpha-2-macroglobulin, with or without AFP-AFPL3-DCP, adjusted for age and gender (patent pending). The cutoff choice for the LCR-glycan algorithm was predetermined as the value giving the highest sum of sensitivity plus specificity.

Sensitivity and specificity for standard tests were assessed in intention to diagnose which included the non-reliable results as failure. Here, failure observed were all due to total serum

haptoglobin 0.1g/L mostly associated with severe cirrhosis. These cases would have been excluded in a per-protocol analysis.

Comparisons used the restricted maximum likelihood estimation-based test statistic, the standard being AFP ≥ 20 ng/mL, the primary endpoint for comparing sensitivity and AUROCs. For AFP-L3 and DCP the standard recommended cutoffs were also used, $\geq 10\%$ of total AFP for AFP-L3 and ≥ 150 mAU/mL for DCP.

Area under the Receiver Characteristic Operating Curves (AUROCs) were assessed using the non-parametric method. All statistical analyses were performed using NCSS-12.0 [30].

Results

N-Glycan profiles of haptoglobin

Patients' characteristics at inclusion according to the presence of cirrhosis and HCC are summarized in Table 1 and the schematic N-glycan profiling workflow for serum haptoglobin is shown in supporting information Figure S1.

According to previous results [15–17] a total of eight glycan structures were identified (Supplementary Table S2).

For the first time our results observed the presence of bifucosylated tetra-antennary glycan (m/z 3316.69), with both core and antennary fucosylation, in HCC patients irrespective of the presence or not of cirrhosis (Figure 2A), suggesting that this N-glycan structure may be a distinctive marker for hepatocellular carcinoma regardless of the presence of cirrhosis.

In order to confirm the haptoglobin N-glycan profile of healthy subjects, we performed the sample preparation and MALDI-TOF analyses on a commercial native human haptoglobin protein. Six N-glycan were identified including nonfucosylated bi-antennary, tri-antennary, and tetra-antennary glycan (m/z 2070.07, 2519.28, 2968.49, respectively) and monofucosylated bi-antennary, tri-antennary, and tetra-antennary glycan (m/z 2244.13, 2693.40, 3142.69, respectively) (Supplementary Figure S2).

We also observed for the first time the haptoglobin N-glycan profiles in patients without HCC but with different stage of liver fibrosis/cirrhosis. In patients without fibrosis (F0) or with minimal fibrosis (F1) (Figure 2B) and in those with significant (F2) or advanced (F3) liver fibrosis (Figure 2C) a N-glycan profile similar to that observed for the native human haptoglobin protein (healthy control), including the six typical glycan, was observed, suggesting that alterations in the protein fucosylation profile are not directly related to liver fibrosis.

Finally, in patients with cirrhosis, not matter if minimal or advanced, a bifucosylated tri-antennary structure (m/z 2867.48) was also observed (Figure 2D).

A schematic representation of haptoglobin N-glycan profile in each liver class is shown in Supplementary Table S3.

Sensitivity and specificity analysis of bifucosylated tetra-antennary glycan for the diagnostic of HCC according to fibrosis/cirrhosis stage

The reliability, sensitivity and specificity of bifucosylated tetra-antennary glycan (m/z 3316.69) in the different populations according to the stage of fibrosis, age and gender are presented in Table 2.

Cases and controls without cirrhosis—In the 21 patients with HCC the intention-to-diagnosis analyses showed that the bifucosylated tetra-antennary glycan had a reliability of 100% (95%CI) and a sensitivity of 71% (15/21;95%CI 50–86) significantly better ($P=0.02$) than standard AFP (43%;9/21;95%CI 24–63). In the 81 controls without HCC, the bifucosylated tetra-antennary glycan had a reliability of 99% (80/81;95%CI 93–100) with a specificity of 96% (78/81;95%CI 90–99), on intention-to-diagnose analysis and 98% (78/80;95%CI 91–100) per protocol. Not significant differences ($P=0.16$) were observed between the specificity of bifucosylated tetra-antennary glycan and standard AFP (99%;80/81;95%CI 99–100).

Cases and controls with cirrhosis—In the 81 patients with HCC, the reliability of bifucosylated tetra-antennary glycan was 70% (57/81;95%CI 59–80). The sensitivity on intention-to-diagnose was 53% (43/81;95%CI 42–64), significantly better ($P=0.03$) than standard AFP (36%;29/81; 95%CI 26–47), and 75% (43/57; 95%CI 62–86) per protocol. In the 59 controls, a reliability of 68% (40/59;95%CI 54–79), a specificity of 46% (27/59;95%CI 33–59) on intention-to-diagnose analysis and 68% (27/40;95%CI 51–81) per protocol were observed.

All 242 cases—The sensitivity and specificity of bifucosylated tetra-antennary glycan alone, based on an intention-to-diagnose analysis (including low haptoglobin as failure), were 57% (58/102 95%CI 47–67) and 75% (105/140;95%CI 67–82) respectively and per protocol (not applicable excluded) were 74% (58/78;95%CI 63–84), and 88% (105/120;95%CI 81–93) respectively.

Sensitivity and specificity analysis of bifucosylated tri-antennary glycan for the diagnostic of HCC according to fibrosis/cirrhosis stage

Patients with cirrhosis, irrespective of whether or not they had HCC, also displayed a bifucosylated tri-antennary structure (m/z 2867.48).

The reliability, sensitivity and specificity of the bifucosylated tri-antennary glycan (m/z 2867.48) in HCC and control populations according to the stage of fibrosis, age and gender are shown in Supplementary Table S4. This glycan had a smaller sensitivity in patients without cirrhosis, without higher specificity in the controls. In cases and controls with cirrhosis, the performances of the tri-antennary glycan was similar to those of the tetra-antennary glycan.

In the 21 patients with HCC without cirrhosis, the bifucosylated tri-antennary structure had a reliability of 100% (95%CI) and a sensitivity of 33% (7/21;95%CI 15–57) using intention-to-diagnose analysis, twice less ($P=0.01$) than the sensitivity of bifucosylated tetra-antennary

glycan 71% (15/21;95%CI 48–89), without better specificity ($P=0.19$). In the 81 controls specificity was 93% (74/80;95%CI 87–98) per protocol and 91% (74/81;95%CI 83–96) in intention to diagnose.

Construction of LCR-glycan algorithm

An algorithm (LCR-glycan) was constructed combining all components of LCR-1 test, total haptoglobin (whatever the serum levels), gammaglutamyl-transpeptidase, apolipoprotein A1, alpha-2-macroglobulin, with AFP, AFPL3, DCP and bifucosylated tetra-antennary glycan, adjusted for age and gender. The sensitivity of LCR-glycan in patients without cirrhosis was 86% (18/21; 95%CI 63–95) significantly better ($P=0.001$) than standard AFP (43%; 9/21; 95%CI 24–63) without significant differences for specificity ($P=0.16$). Comparisons of sensitivities and specificities of bifucosylated tetra-antennary glycan, LCR-glycan, AFP-L3 and DCP versus standard AFP in subset without cirrhosis are showed in Table 3 Panel A.

The AUROC (95%CI) of LCR-glycan for HCC detection in patients without cirrhosis was 0.943 (0.806–0.984) compared to 0.811 (0.630–0.908) for standard AFP ($P=0.06$) (Table 3, Panel B). Similar results were obtained among patients with cirrhosis, with an AUROC (95%CI) of 0.889 (0.821–0.931) for LCR-glycan significantly better ($P<0.001$) than standard AFP (0.750; 0.652–0.823) (Table 3, Panel C).

The performances of LCR-glycan, LCR-1, standard AFP, AFP-L3 and DCP for the diagnosis of liver cancer in all 242 patients are showed in Table 3, Panel C. The AUROC's of LCR-glycan and bifucosylated tetra-antennary glycan were not different according to the type of liver disease (Supplementary Table S5a) or ethnicity (Supplementary Table S5b).

AFP, AFP-L3, DCP in HCC and controls

As shown in Figure 3 we measured serum levels of standard HCC markers, AFP (3A), AFP-L3 (3B), and DCP (3C) according to the stages of liver fibrosis (F0, F1, F2, F3), cirrhosis severity (F4.1, F4.2, F4.3) and HCC.

A marked increase in the three markers levels was found, especially in patients with advanced cirrhosis (F4.3) and HCC, confirming that their serum concentrations were related to both the severity of cirrhosis and to the presence of HCC. Sensitivity and specificity of standard biomarkers (AFP, AFP-L3 DCP) at recommended cutoffs in the different populations are showed in Table 1.

Clinical case

As shown in Table 2 the presence of the bifucosylated tetra-antennary glycan was also observed in 13 patients with cirrhosis without HCC and in 2 patients without cirrhosis or HCC. In one of these 13 controls with cirrhosis, a Caucasian woman, 54 years old at inclusion with cirrhosis (F4.1) due to HCV infection with virological sustained response, 18 months after detection of the isolated bifucosylated tetra-antennary glycan (Supplementary Figure S3) (absence of nodules, AFP, AFP-L3 and DCP levels were normal 6.3 ng/mL, 0.1% and 23 mUA/mL respectively) a moderately differentiated (25 mm size) HCC was identified

in segments V and VI, confirmed by biopsy. Despite segmentectomy, 6 months later a multifocal relapse occurred with an AFP value of 241.100 ng/mL and death.

Discussion

Twenty percent of HCCs may develop on a non-cirrhotic liver, notably in patients with NAFLD, including subjects who are obese or with type 2 diabetes [31–33]. These patients are not included in standard surveillance, as a consequence, these HCCs tend to be discovered later and respond less to anti-cancer therapies. Therefore, there is an urgent need to develop and validate new HCC biomarkers especially in patients without cirrhosis, in order to increase the number of subjects diagnosed with early stage cancer.

Recent evidence indicates that alterations in glycan structure and composition are directly related to hepatocellular malignant transformation and cancer progression. In particular, high levels of bifucosylated tetra-antennary haptoglobin glycan have been identified in patients with early stage HCC [15–17]. Despite the encouraging results, in the previous studies HCC patients have never been stratified into those with and without cirrhosis, making it impossible to determine if the altered expression of this glycan was only correlated to a greater prevalence of cirrhosis in these subjects.

For all these reasons the primary goal of this study was to assess if bifucosylated tetra-antennary glycan could be a marker of hepatocellular carcinoma in patients without cirrhosis.

Our results showed the presence of bifucosylated tetra-antennary structure (m/z 3316.69), with both core and antennary fucosylation, in patients with HCC irrespective of the presence or not of cirrhosis.

Indeed, our HCC population included 20.6% (21/102) of patients without cirrhosis (2 F0, 4 F1, 5 F2, 10 F3). In the non-cirrhotic group 21 patients had HCC, including 10 with F3 stage which in Europe, but not in USA, have a follow up similar to that of cirrhotic patients. In the leaving 11 patients were F0, F1 or F2. We acknowledged the limited number of cases, but in this population with HCC the reliability of bifucosylated tetra-antennary glycan, not limited by low haptoglobin levels due to cirrhosis, was 100% (95%CI) with a sensitivity of 71% (95%CI 48–89), significantly better ($P=0.02$) than standard AFP, and a specificity of 96% (95%CI 91–100) in controls.

These results provide further support to the view that haptoglobin bifucosylated tetra-antennary glycan is strongly related to the process of carcinogenesis regardless of the presence of cirrhosis.

The second aim of our study was to construct a new liver-cancer multi-analyte blood and assess whether this would increase the performance of standard AFP, AFP-L3, DCP. The LCR-glycan algorithm, combining the four components of LCR-1 test (haptoglobin, gammaglutamyl-transpeptidase, apolipoprotein A1, alpha-2-macroglobulin) with AFP, AFP-L3, DCP and haptoglobin bifucosylated tetra-antennary glycan adjusted for age and gender, showed a sensitivity of 86% in patients with HCC and without cirrhosis, significantly better

($P=0.001$) than the one observed for standard AFP, with a AUROC (95%CI) of 0.943 (0.806–0.984) compared to 0.811 (0.630–0.908) for standard AFP ($P=0.06$).

Bifucosylated tetra-antennary glycan was also observed in 13 cirrhotic patients without HCC, and in 2 patients without cirrhosis or HCC (Table 2) suggesting false positives. However, in the absence of longitudinal follow-up, the presence of very early undetectable HCC cannot be excluded. The presence of bifucosylated tetra-antennary glycan could be due to its association with inflammation, first in cirrhosis and then in the presence of the HCC [34,35], which is not in itself a ‘hallmark of cancer’, but plays a significant role in all recognized cancer hallmarks, such as cell dissociation and invasion, cell signaling, metastases and immune modulation [36].

For proof-of-concept, we followed the clinical course of one cirrhotic patient who had no detectable HCC at inclusion, as well as normal AFP, AFP-L3 and DCP values but whose serum haptoglobin N-glycan profile was characterized by the presence of bifucosylated tetra-antennary glycan. The follow-up showed that eighteen months later this patient developed HCC, suggesting that alterations in the haptoglobin N-glycan profile may occur during carcinogenesis, and precede the appearance of a nodule. Longitudinal follow-up in a large population is needed to address the value of bifucosylated tetra-antennary glycan as a very early marker of HCC.

In this study we also observed for the first time the haptoglobin N-glycan profiles in the different seven stages of liver fibrosis/cirrhosis, established according to predetermined FibroTest cutoffs [23]. The diagnostic and prognostic values of FibroTest have been both extensively validated for patients with chronic hepatitis C [23,37], chronic hepatitis B [38, 39], alcoholic liver disease [40,41] and NAFLD [42,43].

Our results showed a profile of six N-glycan in patients with or without liver fibrosis, either minimal or advanced, suggesting that the fucosylated profile of haptoglobin, is not affected by liver fibrosis. Patients with cirrhosis, irrespective of whether or not they had HCC, also displayed a bifucosylated tri-antennary structure (m/z 2867.48). Among patients with HCC, this structure was mostly found in patients with cirrhosis, with a sensitivity of only 33% in the 21 patients with HCC in the absence of cirrhosis, suggesting that this glycan is more related to cirrhosis than to cancer.

Despite these encouraging results the present study has several limitations, including the transversal and retrospective design, the relatively small number of serum specimens and the case control design, which cannot be used to determine the real prevalence of HCC. Therefore, only the sensitivity and the specificity could be assessed. Furthermore, despite the reliability > 95% in patients without cirrhosis, the reliability was only 76% in patients with cirrhosis and HCC and 86% in patients with cirrhosis without HCC, which was due to low serum haptoglobin concentrations. The analytical method used was also complex, time consuming and with a higher current cost compared to standard AFP alone.

Thus, a prospective study is needed that includes more patients from different hospitals to perform and specify how long the alterations in the haptoglobin glycan profile appear before

the appearance of a suspicious nodule as well as to develop a new, simpler, reproducible and low cost method to identify the presence of the target glycan, even at low concentrations.

In conclusion our proof of concept study showed that bifucosylated tetra-antennary glycan is strongly associated with HCC not only in patients with cirrhosis, but also in patients without cirrhosis. Among these patients without cirrhosis there were HCV sustained virologic responders and NAFLD patients, emerging populations at risk of HCC. Combination of bifucosylated tetra-antennary glycan with other biomarkers improves the performance of current HCC biomarkers and recent liver-cancer multi-analyte blood tests for very early detection of liver cancer risk.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations:

HCC	hepatocellular carcinoma
AFP	alpha-Fetoprotein
AFP-L3	lectin-reactive alpha-fetoprotein
DCP	des-gamma-carboxy prothrombin
LCR-1-test	Liver-Cancer-Risk-test
BiFc-tetra-glycan	bifucosylated tetra-antennary haptoglobin glycan
ApoA1	apolipoprotein
Hp	haptoglobin
GGT	gammaglutamyl-transpeptidase

References:

- [1]. Kanwal F, Singal AG. Surveillance for HCC: Current Best Practice and Future Direction. *Gastroenterology* 2019.
- [2]. White DL, Thrift AP, Kanwal F, Davila J, El-Serag HB. Incidence of hepatocellular carcinoma in all 50 United States, from 2000 through 2012. *Gastroenterology* 2017;152:812–820. [PubMed: 27889576]
- [3]. Bruix J, Sherman M. Management of hepatocellular carcinoma: an update. *Hepatology* 2011;53:1020–1022. [PubMed: 21374666]
- [4]. EASL. Practice guidelines: management of hepatocellular carcinoma. *J Hepatol* 2018;69:182–236. [PubMed: 29628281]

- [5]. Flores A, Marrero JA. Emerging trends in hepatocellular carcinoma: focus on diagnosis and therapeutics. *Clin Med Insights Oncol* 2014;8:71–76. [PubMed: 24899827]
- [6]. Ruoslahti E, Salaspuro M, Pihko H, Andersson L, Seppala M. Serum alpha-fetoprotein: diagnostic significance in liver disease. *Br Med J* 1974;2:527–529. [PubMed: 4407283]
- [7]. Di Bisceglie AM, Hoofnagle JH. Elevations in serum alpha-fetoprotein levels in patients with chronic hepatitis B. *Cancer* 1989;64:2117–2120. [PubMed: 2478280]
- [8]. Marrero JA, Kulik LM, Sirlin C, Zhu AX, Finn RS, Abecassis MM, et al. Diagnosis, staging, and management of hepatocellular carcinoma: 2018 Practice Guidance by the American Association for the Study of Liver Diseases. *Hepatology* 2018;68:723–750. [PubMed: 29624699]
- [9]. Poté N, Cauchy F, Albuquerque M, Voitot H, Belghiti J, Castera L, et al. Performance of DCP for early hepatocellular carcinoma diagnosis and prediction of microvascular invasion. *J Hepatol* 2015;62:848–54. [PubMed: 25450201]
- [10]. Marrero JA, Feng Z, Wang Y, Nguyen MH, Befeler AS, Roberts LR, et al. Alpha-fetoprotein, des-gamma carboxyprothrombin, and lectin-bound alpha-fetoprotein in early hepatocellular carcinoma. *Gastroenterology* 2009;137:110–118. [PubMed: 19362088]
- [11]. Zhao YP, Xu XY, Fang M, Wang H, You Q, Yi CH, et al. Decreased core-fucosylation contributes to malignancy in gastric cancer. *PLoS One* 2014;9(4):e94536. [PubMed: 24732908]
- [12]. Osuga T, Takimoto R, Ono M, Hirakawa M, Yoshida M, Okagawa Y, et al. Relationship Between Increased Fucosylation and Metastatic Potential in Colorectal Cancer. *J Natl Cancer Inst* 2016;108(8).
- [13]. Miyoshi E, Moriwaki K, Terao N, Tan CC, Terao M, Nakagawa T, et al. Fucosylation is a promising target for cancer diagnosis and therapy. *Biomolecules* 2012;2(1):34–45. [PubMed: 24970126]
- [14]. Choi JY, Jung SW, Kim HY, Kim M, Kim Y, Kim DG, et al. Diagnostic value of AFP-L3 and DCP in hepatocellular carcinoma according to total-AFP. *World J Gastroenterol* 2013;19(3):339–46. [PubMed: 23372355]
- [15]. Zhang S, Shu H, Luo K, Kang X, Zhang Y, Lu H, et al. N-linked glycan changes of serum haptoglobin beta chain in liver disease patients. *Mol BioSyst* 2011;7:1621–1628. [PubMed: 21380457]
- [16]. Huang Y, Zhou S, Zhu J, Lubman DM, Mechref Y. LC-MS/MS isomeric profiling of permethylated N-glycan derived from serum haptoglobin of hepatocellular carcinoma (HCC) and cirrhotic patients. *Electrophoresis* 2017;38(17):2160–2167. [PubMed: 28543513]
- [17]. Zhang Y, Zhu J, Yin H, Marrero J, Zhang XX, Lubman DM. ESI-LC-MS Method for Haptoglobin Fucosylation Analysis in Hepatocellular Carcinoma and Liver Cirrhosis. *J Proteome Res* 2015;14(12):5388–95. [PubMed: 26503433]
- [18]. Zhu J, Lin Z, Wu J, Yin H, Dai J, Feng Z, et al. Analysis of serum haptoglobin fucosylation in hepatocellular carcinoma and liver cirrhosis of different etiologies. *J Proteome Res* 2014;13(6):2986–97. [PubMed: 24807840]
- [19]. Poynard T, Peta V, Deckmyn O, Munteanu M, Moussalli J, Ngo Y, et al. LCR1 and LCR2, two multi-analyte blood tests to assess liver cancer risk in patients without or with cirrhosis. *Aliment Pharmacol Ther* 2018;49(3):308–320. [PubMed: 30569507]
- [20]. Borlak J, Chatterji B, Londhe KB, Watkins PB. Serum acute phase reactants hallmark healthy individuals at risk for acetaminophen-induced liver injury. *Genome Med* 2013;5:86. [PubMed: 24070255]
- [21]. Peta V, Tse C, Perazzo H, Munteanu M, Ngo Y, Ngo A, et al. Serum apolipoprotein A1 and haptoglobin, in patients with suspected drug-induced liver injury (DILI) as biomarkers of recovery. *PlosOne* 2017;12:e0189436.
- [22]. Xia J, Song P, Sun Z, Sawakami T, Jia M, Wang Z. Advances of diagnostic and mechanistic studies of γ -glutamyl transpeptidase in hepatocellular carcinoma. *Drug Discov Ther* 2016;10:181–187. [PubMed: 27534452]
- [23]. Poynard T, Vergniol J, Ngo Y, Foucher J, Munteanu M, Merrouche W, et al. Staging chronic hepatitis C in seven categories using fibrosis biomarker (FibroTest™) and transient elastography (FibroScan®). *J Hepatol* 2014;60(4):706–14. [PubMed: 24291240]

- [24]. Houot M, Ngo Y, Munteanu M, Marque S, Poynard T. Systematic review with meta-analysis: direct comparisons of biomarkers for the diagnosis of fibrosis in chronic hepatitis C and B. *Aliment Pharmacol Ther* 2016;43(1):16–29. [PubMed: 26516104]
- [25]. Mazzaferro V, Regalia E, Doci R, Andreola S, Pulvirenti A, Bozzetti F, et al. Liver transplantation for the treatment of small hepatocellular carcinomas in patients with cirrhosis. *N Engl J Med* 1996;334:693–699. [PubMed: 8594428]
- [26]. Zhu JH, Wu J, Yin HD, Marrero J, Lubman DM. Mass spectrometric N-glycan analysis of haptoglobin from patient serum samples using a 96-well plate format. *J Proteome Res* 2015;14(11):4932–9. [PubMed: 26448449]
- [27]. Zhu JH, Lin ZX, Wu J, Yin HD, Dai JL, Feng ZD, et al. Analysis of Serum Haptoglobin Fucosylation in Hepatocellular Carcinoma and Liver Cirrhosis of Different Etiologies. *J Proteome Res* 2014;13:2986–2997. [PubMed: 24807840]
- [28]. Kang P, Mechref Y, Klouckova I, Novotny MV. Solidphase permethylation of glycan for mass spectrometric analysis. *Rapid Commun. Mass Spectrom* 2005;19:3421–3428. [PubMed: 16252310]
- [29]. Marrero JA, Feng Z, Wang Y, Nguyen MH, Befeler AS, Roberts LR et al. Alpha-fetoprotein, des-gamma carboxyprothrombin, and lectin-bound alpha-fetoprotein in early hepatocellular carcinoma. *Gastroenterology* 2009;137(1):110–8. [PubMed: 19362088]
- [30]. Hintze JL. NCSS 2011 User Guide Number Cruncher Statistical Systems software NCSS, Kaysville, Utah 2011.
- [31]. Trevisani F, Frigerio M, Santi V, Grignaschi A, Bernardi M. Hepatocellular carcinoma in non-cirrhotic liver: a reappraisal. *Dig Liver Dis* 2010;42(5):341–7. [PubMed: 19828388]
- [32]. White DL, El-Serag HB. Association between nonalcoholic fatty liver disease and risk for hepatocellular cancer, based on systematic review. *Clin Gastroenterol Hepatol* 2012;10:1342–1359. [PubMed: 23041539]
- [33]. Dyson J, Jaques B, Chattopadhyay D, Lochan R, Graham J, Das D, et al. Hepatocellular cancer: the impact of obesity, type 2 diabetes and a multidisciplinary team. *J Hepatol* 2014;60:110–117. [PubMed: 23978719]
- [34]. Arnold JN, Saldova R, Hamid UM. Evaluation of the serum N-linked glycome for the diagnosis of cancer and chronic inflammation. *Proteomics* 2008;16:3284–3293.
- [35]. Comunale MA, Rodemich-Betesh L, Hafner J, Wang M, Norton P, Di Bisceglie AM, et al. Linkage specific fucosylation of alpha-1-antitrypsin in liver cirrhosis and cancer patients: implications for a biomarker of hepatocellular carcinoma. *PLoS One* 2010; 5(8):e12419. [PubMed: 20811639]
- [36]. Munkley J, Elliott DJ. Hallmarks of glycosylation in cancer. *Oncotarget* 2016;7:35478–35489. [PubMed: 27007155]
- [37]. Imbert-Bismut F, Ratziu V, Pieroni L, Charlotte F, Benhamou Y, Poynard T. Biochemical markers of liver fibrosis in patients with hepatitis C virus infection: a prospective study. *Lancet* 2001;357(9262):1069–75. [PubMed: 11297957]
- [38]. Poynard T, Vergniol J, Ngo Y, Foucher J, Thibault V, Munteanu M et al. Staging chronic hepatitis B into seven categories, defining inactive carriers and assessing treatment impact using a fibrosis biomarker (FibroTest®) and elastography (FibroScan®). *J Hepatol* 2014;61(5):994–1003. [PubMed: 25016224]
- [39]. Xu XY, Kong H, Song RX, Zhai YH, Wu XF, Ai WS, et al. The effectiveness of noninvasive biomarkers to predict hepatitis B-related significant fibrosis and cirrhosis: a systematic review and meta-analysis of diagnostic test accuracy. *PLoS One* 2014;9(6):e100182. [PubMed: 24964038]
- [40]. Thiele M, Madsen BS, Hansen JF, Detlefsen S, Antonsen S, Krag A. Accuracy of the Enhanced Liver Fibrosis Test vs FibroTest, Elastography, and Indirect Markers in Detection of Advanced Fibrosis in Patients With Alcoholic Liver Disease. *Gastroenterology* 2018 4;154(5):1369–1379. [PubMed: 29317276]
- [41]. Naveau S, Gaudé G, Asnacios A, Agostini H, Abella A, Barri-Ova N, et al. Diagnostic and prognostic values of noninvasive biomarkers of fibrosis in patients with alcoholic liver disease. *Hepatology* 2009;49(1):97–105. [PubMed: 19053048]

- [42]. Munteanu M, Tiniakos D, Anstee Q, Charlotte F, Marchesini G, Bugianesi E, et al. Diagnostic performance of FibroTest, SteatoTest and ActiTest in patients with NAFLD using the SAF score as histological reference. *Aliment Pharmacol Ther* 2016;44(8):877–89. [PubMed: 27549244]
- [43]. Munteanu M, Pais R, Peta V, Deckmyn O, Moussalli J, Ngo Y, et al. Long-term prognostic value of the FibroTest in patients with non-alcoholic fatty liver disease, compared to chronic hepatitis C, B, and alcoholic liver disease. *Aliment Pharmacol Ther* 2018; 48(10):1117–1127. [PubMed: 30334263]

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Figure 1:
Flow sheet of population subsets

Figure 2:

Representative MALDI-TOF spectra of desialylated haptoglobin N-glycans in sera of patients with HCC (A), without (B) or with (C) liver fibrosis and with cirrhosis (D). A total of 8 glycan structures were identified. Six glycan, including bi-, tri-, and tetra-antennary nonfucosylated (m/z 2070.07, 2519.28, 2968.49) and monofucosylated glycan (m/z 2244.13, 2693.40, 3142.69) were observed in patients with or without liver fibrosis. In cirrhotic patients a tri-antennary glycan (m/z 2867.48) was observed, and in HCC patients, irrespective of the presence or not of cirrhosis, a bifucosylated tetra-antennary glycan (m/z 3316.69) was observed.

Figure 3: Box plots comparing serum levels of AFP (A), AFP-L3 (B) and DCP (C) according to fibrosis stage, cirrhosis severity (F4.1, F4.2, F4.3) and HCC.

Table 1:

Characteristics at inclusion of subset without cirrhosis vs subset with cirrhosis according to the presence or not of HCC.

Characteristics	Subset without cirrhosis n=102		Subset with cirrhosis n=140	
	Cases with HCC	Controls without HCC	Cases with HCC	Controls without HCC
N	21	81	81	59
Age median	62.3	56.9	61.7	64.1
Gender male n(%;95%CI)	16 (76;53–92)	40 (49;38–61)	69 (85;76–92)	47 (80;67–89)
Ethnic background n(%;95%CI)				
Caucasian	14 (67;43–85)	46 (57;45–68)	64 (79;69–87)	39 (66;53–78)
Subsaharian	2 (9;1–30)	12 (15;8–24)	5 (6;2–14)	5 (8;3–19)
NorthAfrican	4 (19;5–42)	14 (17;10–27)	6 (7;3–15)	10 (17;8–29)
Asian	1 (5;0–2)	9 (11;5–20)	6 (7;3–15)	5 (8;3–19)
Liver disease[†] n(%;95%CI)				
ALD	4 (19;5–42)	0	14 (17;10–27)	1 (2;0–9)
CHB	5 (24;8–47)	15 (19;11–29)	16 (20;12–30)	4 (7;2–16)
CHC	6 (29;11–52)	26 (32;22–43)	35 (43;32–55)	36 (61;47–73)
NAFLD	4 (19;5–42)	30 (37;27–48)	8 (10;4–19)	13 (22;13–35)
Others and mixed	2 (9;1–30)	10 (12;6–22)	8 (10;4–19)	5 (8;3–19)
Stage (FibroTest) n(%;95%CI)				
F0 (0.00–0.27)	2 (9;1–30)	27 (34;23–45)	0 (0)	0 (0)
F1 (0.27–0.48)	4 (19;5–42)	14 (17;10–27)	0 (0)	0 (0)
F2 (0.48–0.58)	5 (24;8–47)	21 (26;17–37)	0 (0)	0 (0)
F3 (0.58–0.74)	10 (48;26–70)	19 (23;15–34)	0 (0)	0 (0)
F4.1 (0.74–0.85)	0 (0)	0 (0)	21 (26;17–37)	19 (32;21–46)
F4.2 (0.85–0.95)	0 (0)	0 (0)	36 (44;33–56)	19 (32;21–46)
F4.3 (0.95–1.00)	0 (0)	0 (0)	24 (30;20–41)	21 (36;24–49)
AFP >= 20 ng/mL				
Se n (%;95%CI)	9(43;24–63)		29(36;25–47)	
Sp n (%;95%CI)		80(99;96–100)		51(86;75–94)
AFP-L3 >= 10%				
Se Sp n (%;95%CI)	8(38;21–59)		28(35;24–46)	
Sp n (%;95%CI)		81(100;95–100)		51(86;75–94)
DCP>=150 mAU/mL				
Se n (%;95%CI)	15(71;50–86)		52(64;53–75)	
Sp n (%;95%CI)		78(96;90–99)		46(78;65–88)

[†] ALD, alcoholic liver disease; CHB, chronic hepatitis B; CHC, chronic hepatitis C; NAFLD, Non-alcoholic fatty liver diseases

Table 2:

Reliability, sensitivity (Se), specificity (Sp) of bifucosylated tetra-antennary glycan according to fibrosis stage, cirrhosis severity (F4.1, F4.2, F4.3), age and gender.

Fibrosis stage	Cancer: BiFc-tetra-glycan reliability and sensitivity (Se)					Controls: reliability and specificity (Sp)				
		Reliable	True +	Age 50 y	Male		Reliable	True -	Age 50y	Male
	n	n (%)	n (Se)	n (%)	n (%)	n	n (%)	n (Sp %)	n (%)	n (%)
F0	2	2 (100)	1 (50)	1 (50)	1(50)	27	27 (100)	27 (100)	12(44)	12(44)
F1	4	4 (100)	3 (75)	4 (100)	4 (100)	14	14 (100)	13 (93)	5 (36)	5(36)
F2	5	5 (100)	4 (80)	2 (5)	2 (40)	21	21 (100)	21 (100)	12(57)	12(57)
F3	10	10 (100)	7 (70)	9 (90)	9 (90)	19	18 (95)	17 (89)	11(58)	11(58)
<i>Subtotal noF4</i>	<i>21</i>	<i>21 (100)</i>	<i>15 (71)</i>			<i>81</i>	<i>80 (99)</i>	<i>78 (96)</i>		
F4.1	21	19 (88)	13 (62)	16 (21)	16 (76)	19	15 (79)	11 (58)	16(84)	16(84)
F4.2	36	25 (69)	18 (50)	31 (86)	31 (86)	19	11 (58)	9 (47)	17(90)	17(90)
F4.3	24	13 (54)	12 (46)	22 (24)	22 (92)	21	14 (67)	7 (33)	14(67)	21(68)
<i>Subtotal F4</i>	<i>81</i>	<i>57 (70)</i>	<i>43 (53)</i>			<i>59</i>	<i>40 (68)</i>	<i>27(46)</i>		
Total	102	78 (76)	58 (57) *	91 (89) **	85 (83) *	140	120 (86)	105 (75)	87(62)	87(62)
CI 95%		(68–85)	(47–66)	(83–95)	(76–91)		(80–92)	(68–82)	(54–70)	(54–70)

NA: not reliable Hp < 0.1 g/L.

* P<0.001 between HCC and controls

** P=0.005 between HCC and controls

Table 3:

Sensitivity, specificity and performances (AUROC) of LCR-glycans, BiFc-tetra-glycan, LCR-1, AFP-L3 and DCP vs standard AFP.

Panel A: Comparisons of sensitivities and specificities in subset without cirrhosis

Criterion	Sensitivity	(95%CI)	P-value	Specificity	(95%CI)	P-value
AFP (standard)	43	24–63	Vs. AFP	99	96–100	Vs. AFP
BiFc-tetra-glycan	71	50–86	0.02	96	90–99	0.16
LCR-glycan	86	63–95	0.001	96	90–99	0.16
AFP-L3	38	21–59	0.67	100	95–100	0.84
DCP	71	50–86	0.02	96	90–99	0.16

Panel B: subset without cirrhosis

Criterion	Number patients	AUROC	95% Confidence Limits				
			>0.5	P-value	Lower	Upper	P-value Vs. AFP
AFP (standard)	102	0.811	4.587	<0.001	0.630	0.908	
BiFc-tetra-glycan	102	0.834	6.465	<0.001	0.701	0.911	0.792
LCR-glycan	102	0.943	12.065	<0.001	0.806	0.984	0.064
AFP-L3	102	0.726	3.752	<0.001	0.585	0.825	0.142
DCP	102	0.850	5.191	<0.001	0.653	0.939	0.678
LCR-1	102	0.727	3.491	<0.001	0.573	0.832	0.118

Panel C: subset with cirrhosis

Criterion	Number patients	AUROC	95% Confidence Limits				
			>0.5	P-value	Lower	Upper	P-value Vs. AFP
AFP (standard)	140	0.750	5.777	<0.001	0.652	0.823	
BiFc-tetra-glycan	140	0.590	1.818	0.035	0.485	0.678	0.008
LCR-glycan	140	0.889	14.255	<0.001	0.821	0.931	<0.001
AFP-L3	140	0.726	5.312	<0.001	0.632	0.799	0.461
DCP	140	0.802	7.593	<0.001	0.709	0.868	0.318
LCR-1	140	0.548	0.980	0.164	0.444	0.638	<0.001

Panel D: all 242 patients.

Criterion	Number patients	AUROC	95% Confidence Limits				
			>0.5	P-value	Lower	Upper	P-value Vs. AFP
AFP (standard)	242	0.815	10.771	<0.001	0.749	0.865	
BiFc-tetra-glycan	242	0.749	8.120	<0.001	0.682	0.803	0.0899
LCR-glycan	242	0.932	24.594	<0.001	0.882	0.951	<0.001
AFP-L3	242	0.775	9.633	<0.001	0.712	0.825	0.069
DCP	242	0.855	12.969	<0.001	0.792	0.901	0.288
LCR-1	242	0.729	7.135	<0.001	0.660	0.786	0.009

Restricted maximum likelihood estimation-based test statistic.