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

3
4 Peta Valentina^{1,7}, Jianhui Zhu², David M. Lubman², Samuel Huguet³, Francoise Imbert
5 Bismut⁴, Gérard Bolbach^{5,6}, Gilles Clodic⁵, Lucrece Matheron⁵, Yen Ngo¹, Pais Raluca^{4,7},
6 Chantal Housset⁷, Keyvan Rezaei³, Thierry Poynard^{4,7}

7 1. BioPredictive, Paris, France

8 2. Department of Surgery, University of Michigan Medical Center, Ann Arbor, MI 48019,
9 USA

10 3. Radiopharmacology Department, Institut Curie, Saint Cloud, France

11 4. Assistance Publique-Hôpitaux de Paris (AP-HP), Groupe Hospitalier Pitié-Salpêtrière
12 (GHPS), Paris, France.

13 5. Sorbonne Université, Institut de Biologie Paris Seine, Plate-forme spectrométrie de masse
14 et protéomique, Paris, France

15 6. Sorbonne Université, École normale supérieure, PSL University, CNRS, Laboratoire des
16 Biomolécules (LBM), 75005 Paris, France

17 7. Sorbonne Université, INSERM, Centre de Recherche Saint-Antoine (CRSA), Paris, France.
18 Institutional Author(s): FibroFrance-GHPS

19 * A complete list of investigators in the FibroFrance-Groupe Hospitalier Pitié Salpêtrière
20 (GHPS) Group is provided in **Supporting information File 1**.

21 **Corresponding author:** Thierry Poynard, thierry@poynard.com, +33142161022

22 **Keywords:** Haptoglobin, fucosylation, hepatocellular carcinoma, glycan, cirrhosis

23 **Abbreviations:** HCC, hepatocellular carcinoma; AFP, alpha-Fetoprotein; lectin-reactive
24 alpha-fetoprotein, AFP-L3; des-gamma-carboxy prothrombin, DCP; LCR-1-test, Liver-

25 Cancer-Risk-test; BiFc-tetra-glycan, bifucosylated tetra-antennary haptoglobin glycan;
26 ApoA1, apolipoprotein; Hp, haptoglobin; GGT, gammaglutamyl-transpeptidase.

27 **Conflict of interest statement**

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

31 Valentina Peta and Yen Ngo are full employees of BioPredictive.

32 The other authors have nothing to declare: Chantal Housset, David M. Lubman, Françoise
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39 Experiment conception and design: TP. Experiment performance: VP, SH, JZ, GC, LMD,
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41 Data analysis: TP, VP, YG, DML, RP. Drafting of the paper: TP, VP, CH, KR, DML, GB

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46

47 All authors approved the final version of the manuscript.

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52

53 **Abstract:**

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

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

67 **Results:** In 102 patients without cirrhosis (21 HCC and 81 controls), the intention-to-
68 diagnose analyses showed that haptoglobin bifucosylated tetra-antennary glycan alone had a
69 sensitivity of 71% (15/21;95%CI 50-86), significantly better (P=0.02) than standard AFP
70 (43%;9/21;95%CI 24-63), and a specificity of 96% (78/81;95% 90-99). The sensitivity of
71 LCR-glycan, in patients without cirrhosis, was 86% (18/21; 95%CI 63-95) significantly better
72 (P=0.001) than standard AFP (43%; 9/21; 95%CI 24-63), with an AUROC of 0.943 (95%CI
73 0.806-0.98) compared to 0.811 (95%CI 0.630-0.908) for AFP (P=0.06).

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

78 **Introduction**

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

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

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

99 Des-gamma-carboxy prothrombin (DCP), an abnormal prothrombin protein that is
100 found at higher levels in the serum of HCC patients, has been used as an alternative marker
101 for the diagnosis of HCC [9]. However, the diagnostic value of DCP alone varies depending

102 on patient characteristics [10], and current guidelines only recognize its performance for the
103 stratification of the risk of HCC but do not recommend its use in the surveillance of patients
104 with cirrhosis.

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

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

120 We recently constructed two multi-analyte blood of HCC risk, LCR1 and LCR2, with
121 good performances in patients with chronic liver disease [19]. LCR1 (Live Cancer Risk 1) is
122 a multi-analyte blood test for the early stratification of the risk of cancer, able to identify
123 patients without cirrhosis with a high risk of liver cancer at 10 years. LCR1 combines two
124 proteins associated with liver tissue repair, apolipoprotein A1 (ApoA1) and haptoglobin (Hp)
125 [20,21], three factors previously shown to be associated with HCC risk, gender, age and

126 gammaglutamyl-transpeptidase (GGT) [22] and adjustment for a fibrosis biomarker, alpha2-
127 macroglobulin. LCR2 (Live Cancer Risk 2), combining the components of LCR1 test with
128 AFP, showed better performance than AFP alone for the prediction of the occurrence of
129 cancer at 5 years, both in patients without and with cirrhosis..

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

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

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

141 **Patients and Methods:**

142

143 *Serum samples and study*

144 We retrospectively selected patients with available frozen serum stored at -80°C from
145 2012 to 2015, without previous HCC or liver transplantation. Patients belong to the “Groupe
146 Hospitalier Pitié-Salpêtrière” prospective cohort of FibroFrance, a program that began in 1997
147 (Clinical registry number: NCT01927133). The protocol was approved by the institutional
148 review board, appropriate regulatory agency and performed in accordance with principles of
149 Good Clinical Practice. All patients provided written informed consent before entry.

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

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

164 The diagnosis of HCC was based on non-invasive imaging, including multiphasic
165 computed tomography (CT) and magnetic resonance imaging (MRI), and on histological

166 examination by an experienced pathologist [7,8]. All HCC patients were classified according
167 to Milan criteria staging system [25].

168

169 *Isolation of haptoglobin from serum*

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

177

178 *Deglycosylation and desialylation of haptoglobin*

179 To simplify the glycan spectrum and improve the sensitivity of the method,
180 haptoglobin desialylation was performed. The eluted haptoglobin was denatured by adding 1
181 μ L of denaturing buffer (0.2% sodium dodecyl sulfate, 100 mmol/L β -mercaptoethanol) and
182 incubation at 65°C for 30 min. One unit of peptide N-glycosidase F (PNGase F) (New
183 England Biolabs) was added to release N-glycan. The reaction mixture was incubated at 37°C
184 overnight and quenched by heating at 95°C for 10 min. The mixture was dried, followed by
185 desialylation with 40 mU of neuraminidase (Sigma-Aldrich) at 37°C overnight. The
186 desialylated N-glycan were dried, redissolved in 10 μ L of water (with 0.1% trifluoroacetic
187 acid), and then purified using porous graphitized carbon tips [27].

188

189

190 ***Permethylation of glycan and MALDI-TOF analysis***

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

199

200 ***Measurement of AFP, AFP-L3 and DCP***

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

209

210 ***Biochemical analyses***

211 The FibroTest component assays were performed on an automatic analyzer Modular P
212 from Roche Diagnostics (Mannheim, Germany). Proteins concentrations were measured
213 according to turbidimetric analytical methods using manufacturer's reagents for haptoglobin
214 and ApoA1, and Diagam (Ghislenghien, Belgium) reagents for alpha-2-macroglobulin. GGT

215 was determined using Szasz method and calibrator value given for the international federation
216 of clinical chemistry (IFCC).

217

218 *Construction of LCR-glycan algorithm and statistical analysis*

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

224 Sensitivity and specificity for standard tests were assessed in intention to diagnose
225 which included the non-reliable results as failure. Here, failure observed were all due to total
226 serum haptoglobin ≤ 0.1 g/L mostly associated with severe cirrhosis. These cases would have
227 been excluded in a per-protocol analysis.

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

232 Area under the Receiver Characteristic Operating Curves (AUROCs) were assessed
233 using the non-parametric method. All statistical analyses were performed using NCSS-12.0
234 [30].

235 **Results**

236 *N-Glycan profiles of haptoglobin*

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

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

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

247 In order to confirm the haptoglobin N-glycan profile of healthy subjects, we
248 performed the sample preparation and MALDI-TOF analyses on a commercial native human
249 haptoglobin protein. Six N-glycan were identified including nonfucosylated bi-antennary, tri-
250 antennary, and tetra-antennary glycan (m/z 2070.07, 2519.28, 2968.49, respectively) and
251 monofucosylated bi-antennary, tri-antennary, and tetra-antennary glycan (m/z 2244.13,
252 2693.40, 3142.69, respectively) (**Supplementary Figure S2**).

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

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

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

264

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

267 The reliability, sensitivity and specificity of bifucosylated tetra-antennary glycan (m/z
268 3316.69) in the different populations according to the stage of fibrosis, age and gender are
269 presented in **Table 2**.

270 *Cases and controls without cirrhosis*

271 In the 21 patients with HCC the intention-to-diagnosis analyses showed that the
272 bifucosylated tetra-antennary glycan had a reliability of 100% (95%CI) and a sensitivity of
273 71% (15/21;95%CI 50-86) significantly better (P=0.02) than standard AFP (43%;9/21;95%CI
274 24-63). In the 81 controls without HCC, the bifucosylated tetra-antennary glycan had a
275 reliability of 99% (80/81;95%CI 93-100) with a specificity of 96% (78/81;95%CI 90-99), on
276 intention-to-diagnose analysis and 98% (78/80;95%CI 91-100) per protocol. Not significant
277 differences (P=0.16) were observed between the specificity of bifucosylated tetra-antennary
278 glycan and standard AFP (99%;80/81;95%CI 99-100).

279 *Cases and controls with cirrhosis*

280 In the 81 patients with HCC, the reliability of bifucosylated tetra-antennary glycan
281 was 70% (57/81;95%CI 59-80). The sensitivity on intention-to-diagnose was 53%
282 (43/81;95%CI 42-64), significantly better (P=0.03) than standard AFP (36%;29/81; 95%CI
283 26-47), and 75% (43/57; 95%CI 62-86) per protocol. In the 59 controls, a reliability of 68%

284 (40/59;95%CI 54-79), a specificity of 46% (27/59;95%CI 33-59) on intention-to-diagnose
285 analysis and 68% (27/40;95%CI 51-81) per protocol were observed.

286 *All 242 cases*

287 The sensitivity and specificity of bifucosylated tetra-antennary glycan alone, based on
288 an intention-to-diagnose analysis (including low haptoglobin as failure), were 57% (58/102
289 95%CI 47-67) and 75% (105/140;95%CI 67-82) respectively and per protocol (not applicable
290 excluded) were 74% (58/78;95%CI 63-84), and 88% (105/120;95%CI 81-93) respectively.

291

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

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

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

302 In the 21 patients with HCC without cirrhosis, the bifucosylated tri-antennary structure had a
303 reliability of 100% (95%CI) and a sensitivity of 33% (7/21;95%CI 15-57) using intention-to-
304 diagnose analysis, twice less (P=0.01) than the sensitivity of bifucosylated tetra-antennary
305 glycan 71% (15/21;95%CI 48-89), without better specificity (P=0.19). In the 81 controls
306 specificity was 93% (74/80;95%CI 87-98) per protocol and 91% (74/81;95%CI 83-96) in
307 intention to diagnose.

308

309

310 *Construction of LCR-glycan algorithm*

311 An algorithm (LCR-glycan) was constructed combining all components of LCR-1 test,
312 total haptoglobin (whatever the serum levels), gammaglutamyl-transpeptidase, apolipoprotein
313 A1, alpha-2-macroglobulin, with AFP, AFPL3, DCP and bifucosylated tetra-antennary
314 glycan, adjusted for age and gender. The sensitivity of LCR-glycan in patients without
315 cirrhosis was 86% (18/21; 95%CI 63-95) significantly better (P=0.001) than standard AFP
316 (43%; 9/21; 95%CI 24-63) without significant differences for specificity (P=0.16).
317 Comparisons of sensitivities and specificities of bifucosylated tetra-antennary glycan, LCR-
318 glycan, AFP-L3 and DCP versus standard AFP in subset without cirrhosis are showed in
319 **Table 3 Panel A.**

320 The AUROC (95%CI) of LCR-glycan for HCC detection in patients without cirrhosis
321 was 0.943 (0.806-0.984) compared to 0.811 (0.630-0.908) for standard AFP (P=0.06) (**Table**
322 **3, Panel B**). Similar results were obtained among patients with cirrhosis, with an AUROC
323 (95%CI) of 0.889 (0.821-0.931) for LCR-glycan significantly better (P=<0.001) than standard
324 AFP (0.750; 0.652-0.823) (**Table 3, Panel C**).

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

329

330 *AFP, AFP-L3, DCP in HCC and controls*

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

334

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

340

341 *Clinical case*

342 As shown in **Table 2** the presence of the bifucosylated tetra-antennary glycan was also
343 observed in 13 patients with cirrhosis without HCC and in 2 patients without cirrhosis or
344 HCC. In one of these 13 controls with cirrhosis, a Caucasian woman, 54 years old at inclusion
345 with cirrhosis (F4.1) due to HCV infection with virological sustained response, 18 months
346 after detection of the isolated bifucosylated tetra-antennary glycan (**Supplementary Figure**
347 **S3**) (absence of nodules, AFP, AFP-L3 and DCP levels were normal 6.3 ng/mL, 0.1% and 23
348 mUA/mL respectively) a moderately differentiated (25 mm size) HCC was identified in
349 segments V and VI, confirmed by biopsy. Despite segmentectomy, 6 months later a
350 multifocal relapse occurred with an AFP value of 241.100 ng/mL and death.

351 **Discussion**

352

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

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

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

368 Our results showed the presence of bifucosylated tetra-antennary structure (m/z
369 3316.69), with both core and antennary fucosylation, in patients with HCC irrespective of the
370 presence or not of cirrhosis.

371 Indeed, our HCC population included 20.6% (21/102) of patients without cirrhosis (2
372 F0, 4 F1, 5 F2, 10 F3). In the non-cirrhotic group 21 patients had HCC, including 10 with F3
373 stage which in Europe, but not in USA, have a follow up similar to that of cirrhotic patients.
374 In the leaving 11 patients were F0, F1 or F2. We acknowledged the limited number of cases,
375 but in this population with HCC the reliability of bifucosylated tetra-antennary glycan, not
376 limited by low haptoglobin levels due to cirrhosis, was 100% (95%CI) with a sensitivity of

377 71% (95%CI 48-89), significantly better (P=0.02) than standard AFP, and a specificity of
378 96% (95%CI 91-100) in controls.

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

382 The second aim of our study was to construct a new liver-cancer multi-analyte blood
383 and assess whether this would increase the performance of standard AFP, AFP-L3, DCP. The
384 LCR-glycan algorithm, combining the four components of LCR-1 test (haptoglobin,
385 gammaglutamyl-transpeptidase, apolipoprotein A1, alpha-2-macroglobulin) with AFP, AFP-
386 L3, DCP and haptoglobin bifucosylated tetra-antennary glycan adjusted for age and gender,
387 showed a sensitivity of 86% in patients with HCC and without cirrhosis, significantly better
388 (P=0.001) than the one observed for standard AFP, with a AUROC (95%CI) of 0.943 (0.806-
389 0.984) compared to 0.811 (0.630-0.908) for standard AFP (P=0.06).

390 Bifucosylated tetra-antennary glycan was also observed in 13 cirrhotic patients
391 without HCC, and in 2 patients without cirrhosis or HCC (**Table 2**) suggesting false positives.
392 However, in the absence of longitudinal follow-up, the presence of very early undetectable
393 HCC cannot be excluded. The presence of bifucosylated tetra-antennary glycan could be due
394 to its association with inflammation, first in cirrhosis and then in the presence of the HCC
395 [34,35], which is not in itself a ‘hallmark of cancer’, but plays a significant role in all
396 recognized cancer hallmarks, such as cell dissociation and invasion, cell signaling, metastases
397 and immune modulation [36].

398 For proof-of-concept, we followed the clinical course of one cirrhotic patient who had
399 no detectable HCC at inclusion, as well as normal AFP, AFP-L3 and DCP values but whose
400 serum haptoglobin N-glycan profile was characterized by the presence of bifucosylated tetra-
401 antennary glycan. The follow-up showed that eighteen months later this patient developed

402 HCC, suggesting that alterations in the haptoglobin N-glycan profile may occur during
403 carcinogenesis, and precede the appearance of a nodule. Longitudinal follow-up in a large
404 population is needed to address the value of bifucosylated tetra-antennary glycan as a very
405 early marker of HCC.

406 In this study we also observed for the first time the haptoglobin N-glycan profiles in
407 the different seven stages of liver fibrosis/cirrhosis, established according to predetermined
408 FibroTest cutoffs [23]. The diagnostic and prognostic values of FibroTest have been both
409 extensively validated for patients with chronic hepatitis C [23,37], chronic hepatitis B [38,
410 39], alcoholic liver disease [40,41] and NAFLD [42,43].

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

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

426 Thus, a prospective study is needed that includes more patients from different
427 hospitals to perform and specify how long the alterations in the haptoglobin glycan profile
428 appear before the appearance of a suspicious nodule as well as to develop a new, simpler,
429 reproducible and low cost method to identify the presence of the target glycan, even at low
430 concentrations.

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

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569 **Figure legends**

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571 **Figure 1:** Flow sheet of population subsets

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573 **Figure 2:** Representative MALDI-TOF spectra of desialylated haptoglobin N-glycans in sera
574 of patients with HCC (A), without (B) or with (C) liver fibrosis and with cirrhosis (D). A total
575 of 8 glycan structures were identified. Six glycan, including bi-, tri-, and tetra-antennary
576 nonfucosylated (m/z 2070.07, 2519.28, 2968.49) and monofucosylated glycan (m/z 2244.13,
577 2693.40, 3142.69) were observed in patients with or without liver fibrosis. In cirrhotic
578 patients a tri-antennary glycan (m/z 2867.48) was observed, and in HCC patients, irrespective
579 of the presence or not of cirrhosis, a bifucosylated tetra-antennary glycan (m/z 3316.69) was
580 observed.

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

584 **Table 1:** Characteristics at inclusion of subset without cirrhosis vs subset with cirrhosis
 585 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)
DGP>=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)

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

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

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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)

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NA: not reliable Hp < 0.1 g/L. *P<0.001 between HCC and controls **P=0.005 between HCC and controls

594 **Table 3:** Sensitivity, specificity and performances (AUROC) of LCR-glycans, BiFc-tetra-
 595 glycan, LCR-1, AFP-L3 and DCP vs standard AFP.

596

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

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Criterion	Sensitivity	(95%CI)	P-value	Specificity	(95%CI)	P-value
AFP (standard)	43	24-63	<i>Vs. AFP</i>	99	96-100	<i>Vs. AFP</i>
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

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Restricted maximum likelihood estimation-based test statistic.

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601 **Panel B:** subset without cirrhosis

Criterion	Number patients	AUROC	>0.5	P-value	95% Confidence Limits		P-value <i>Vs. AFP</i>
					Lower	Upper	
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

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604 **Panel C:** subset with cirrhosis

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Criterion	Number patients	AUROC	>0.5	P-value	95% Confidence Limits		P-value <i>Vs. AFP</i>
					Lower	Upper	
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

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618 **Panel D:** all 242 patients.

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Criterion	Number patients	AUROC	>0.5	P-value	95% Confidence Limits		P-value Vs. AFP
					Lower	Upper	
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

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