Title:
Identification of serum proton NMR metabolomic fingerprints associated with hepatocellular carcinoma in patients with alcoholic cirrhosis

Running title:
Serum metabolomics and liver cancer

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Translational Relevance

Hepatocellular carcinoma (HCC) is one of the leading cancers worldwide in terms of incidence and mortality. Most of HCC arise on cirrhosis in western countries, alcohol intake being the main cause of cirrhosis and related HCC in these countries. The difficulties to assess the outcome of such patients stress the need to identify new biomarkers in order to refine the identification of those with a poorer therapeutic response. The present data enabled us to observe changes in serum metabolomic profiles in patients with advanced HCC that reflected the distinct activation or impairment of multiple biological pathways. Furthermore, the analysis small-HCC patients eligible for curative procedures revealed various metabolomic profiles that probably reflect differences in outcome after tumour ablation. By providing new patterns of recognition, serum metabolomic profiling may represent a clinical breakthrough in order to refine prognosis and to guide therapeutic procedures for these patients.
Abstract

Purpose: Metabolomics depicts metabolic changes in biological systems using a multiparametric analysis technique. This study assessed the metabolomic profiles of serum, obtained by proton NMR spectroscopy, from cirrhotic patients with and without hepatocellular carcinoma (HCC). Experimental Design: The study included 154 consecutive patients with compensated biopsy-proven alcoholic cirrhosis. Among these, 93 had cirrhosis without HCC, 28 had biopsy-proven HCC within the Milan criteria and were eligible for curative treatment ('small' HCC) and 33 had HCC outside the Milan criteria ('large' HCC). Proton spectra were acquired at 500 MHz. An orthogonal partial latent structure (OPLS) analysis model was built to discriminate large HCC spectra from cirrhotic spectra. Small HCC spectra were secondarily projected using previously built OPLS discriminant components. Results: The OPLS model showed discrimination between cirrhotic and large HCC spectra. Metabolites that significantly increased with large HCC were, glutamate, acetate and N-acetyl glycoproteins, whereas metabolites that correlated with cirrhosis were lipids and glutamine. Projection of small HCC samples into the OPLS model showed a heterogeneous distribution between large HCC and cirrhotic samples. Small HCC patients with metabolomic profile similar to those of large HCC group had higher incidences of recurrence or death during follow-up. Conclusions: Serum NMR-based metabolomics identified metabolic fingerprints that could be specific to large HCC in cirrhotic livers. From a metabolomic standpoint, some patients with small HCC, who are eligible for curative treatments, seem to behave as patients with advanced cancerous disease. It would be useful to further prospectively investigate these patients to define a subgroup with a worse prognosis.
Introduction

Staging systems of hepatocellular carcinoma (HCC) aim to assess prognosis and to select adequate treatments for each case, particularly in patients eligible for curative procedures, such as hepatic resection, percutaneous ablation or liver transplantation (1). However, there is still a need for biological markers to be associated with clinical outcomes or treatment responses. A definition of the molecular classification of HCC is still preliminary and, unlike other tumours, as yet, no molecular data have been incorporated into algorithms for the routine management of liver cancer (2). A wide spectrum of serum biomarkers has been studied as potential diagnosis or prognosis tools in this setting, but their ability to refine HCC classification or to predict the patient’s outcome is still debated. Consequently, the main prognostic factors used in practice are restricted to clinical features such as tumour status and liver-function impairment linked to the underlying liver disease.

Metabolomics is an ‘omics’ technique that is situated downstream of genomics, transcriptomics and proteomics (3). Proton nuclear magnetic resonance (\(^1\)H NMR) spectroscopy-based metabolomics can identify and quantify metabolic changes within a biological system and has been applied to various pathological conditions (4). The development of metabolomics in the field of oncology may be used to identify fingerprints, profiles or signatures, which could be useful for screening or diagnosis procedures as well as refining prognosis or therapeutic response in various tumors such as prostate, breast, ovarian, or brain cancers(5, 6).

Recent studies have attempted to describe the metabolic phenotype of liver cancer in heterogeneous populations of patients with HCC using \(^1\)H NMR or mass spectroscopy. This has led to the identification of various metabolically impaired pathways in serum and urine (7-10). However, these data were obtained in small sample-sized cohorts of patients and important clinical information was lacking, such as the stage of liver cancer and the cause and
degree of severity of the underlying liver disease, which can induce specific and serious serum metabolic disturbances (11). Furthermore, these studies were mostly restricted to Asian populations in whom cases of HCC were as a whole related to HCV or HBV chronic infections.

The aim of the present study was to assess serum metabolomic profiles in a large cohort of well-characterized Caucasian patients with HCC that developed in alcoholic cirrhotic liver and classified according to their tumor status.
PATIENTS AND METHODS

Patients and collection of serum samples

We considered all out-patients who were consecutively referred to our Hepatology unit for the management of cirrhosis and/or HCC between January 2009 and December 2010, and who fulfilled the following inclusion criteria: 1) biopsy-proven cirrhosis and chronic daily alcohol consumption >50 g; 2) no infection from the human immunodeficiency, hepatitis B or C viruses; 3) compensated liver disease (Child–Pugh score <8); 4) residence in France and of Caucasian origin; and 5) availability of frozen serum samples. Exclusion criteria were 1) treatment of HCC before serum collection; 2) past history or on-going extra-hepatic neoplasm or inflammatory systemic disease; 3) recent history of acute or chronic liver failure in the previous 6 months.

For each patient, the date of inclusion was the date of serum collection. Demographic, clinical and routine biological data were recorded at inclusion. Blood samples were drawn under fasting conditions before therapeutic management of HCC. Sera were separated and stored at –80°C until analysis. All patients gave their written consent for blood sampling. The local ethics committee approved the protocol.

HCC was diagnosed according to the Barcelona criteria (12): histological evidence or convergent demonstration of a focal lesion ≥2 cm in size and arterial hypervascularization as assessed by two different imaging techniques, or the combination of one imaging technique that showed this morphological aspect plus an α-fetoprotein (AFP) level of ≥400 ng/mL in patients with biopsy-proven cirrhosis.

Patients were classified according to the presence and status of HCC as follows.

1) Cirrhosis group: patients with cirrhosis without evidence of HCC at the time of inclusion, as judged by negative ultrasonographic findings, serum AFP of <50 ng/mL. All patients were prospectively evaluated every six months for HCC periodical screening for at least one year.

None of the selected patients in this sub-group developed liver tumour during this time.
2) Small HCC group: patients with cirrhosis and ‘small’ HCC within the Milan criteria (13) (single tumour <5 cm or three nodules <3 cm) and eligible for curative treatment; all these patients underwent radiofrequency ablation (RFA) by the same operator during which a percutaneous biopsy for histological assessment of HCC was performed before ablation. Only patients with biopsy-proven HCC were selected in this subset. All of them were then followed-up using abdominal CT scan, serum alpha-fetoprotein assessment and physical examination at one month then every 3 months. Patients with partial response at one month defined by a persistent enhancement of the lesion on CT scan could have been re-treated by RFA. Follow-up ended at the date of death or liver transplantation, or at the last recorded visit (or information) within the last 6 months before December 2011. Overall survival was defined by the time between the day of inclusion and death, liver transplantation or last visit recorded until December 2011. Recurrence free survival was defined by the time between the date of inclusion and the date of HCC recurrence.

3) Large HCC group: patients with cirrhosis and ‘large’ HCC outside the Milan criteria (multinodular HCC and/or tumoural portal vein thrombosis and/or extra-hepatic metastases), (1) with these patients being eligible for palliative treatment.

\[ \text{\textsuperscript{1}H NMR spectroscopy} \]

For NMR analysis, samples were thawed at room temperature. A volume of 0.6 mL of serum was placed into a 5-mm-diameter specific tube together with 0.1 mL of \( \text{D}_2\text{O} \) containing a known amount of fumaric acid (6.5 mg. mL\(^{-1}\)). The deuterium oxide was used for the spectrometer locking and fumaric signal for chemical shift calibration at 6.53 ppm. The addition of this acid solution lowered the sera pH of 0.1 units (identical for all samples). The resulting pH samples were between 8.4 and 8.7. Fumaric acid as an internal reference presents the advantages to produce a single signal located in a spectral region without other interfering
resonances. The proton spectra were acquired at 500 MHz on a Varian Unity Inova® spectrometer at 298 K. A signal was acquired after a 90° pulse of 32K data points on a spectral window of 5000 Hz. The relaxation delay was 4 s. The water signal was suppressed by a pre-saturation sequence using low-power irradiation (0.03 W for 2 s) on the water-signal frequency during the relaxation delay. Frequently, the CPMG sequence is used to suppress the broad signal of protein according to their short relaxation time. Because the lipid changes in the serum of patients with HCC, we have used a single pulse sequence to preserve the complete lipid profile of our spectra. The resulting free induction decays obtained with 128 transients were processed by Mestrec® software. A Fourier transformation was applied with an exponential window function to produce a 1-Hz broadening line. Spectra were phased and a spline baseline correction was applied with three points at 0.5 and 9 ppm. The spectral region between 0–9 ppm was divided into 9000 spectral regions of 0.001 ppm width, called buckets, using a personal program with R®. Water, urea (signal damaged by water-saturation transfer) and fumaric-acid regions were excluded (1810 variables excluded). Each bucket was integrated and scaled to the total summed integrals for each spectrum.

For resonance assignment purpose, $^1$H-$^1$H TOCSY 2D NMR spectra were also acquired at 298 K for some samples with a mixing time of 80 ms, 4k data points and 32 transients for each of the 512 increments.

**Univariate statistical analyses**

Qualitative variables were compared using Fisher’s exact test, the chi-squared test, or the chi-squared trend test with 1 degree of freedom. Quantitative variables were compared using the non-parametric Wilcoxon test. All reported $P$ values are two-tailed. Associations were considered statistically significant at a two-tailed $\alpha$ of 0.05.
Multivariate statistical analyses
A principal component analysis (PCA) was first performed to detect any outliers or group separation based on NMR-signal variability.

An orthogonal projection to latent-structure (OPLS) analysis was run to discriminate patients from the cirrhosis or large HCC groups. Compared to the classical projection of latent-structure analysis (PLS), this method allowed improved interpretation of the spectroscopic variations between discriminated groups, by removing information that had no impact on discrimination.

Samples were split into three sets: training set, test set and small HCC set. The training set was used to build the OPLS model. The test set was projected on the OPLS model for validation and to assess the general predictive power of the model.

The training and test sets were randomly constituted of 84 training cases (62 cirrhosis and 22 large HCC) and 42 test cases (31 cirrhosis and 11 large HCC). The small HCC set included 28 small HCC cases and the same 31 cirrhosis cases used in the test set.

The goodness-of-fit parameters of the OPLS model, $R^2X$, $R^2Y$ and $Q^2Y$, were calculated. $R^2X$ and $R^2Y$ represent the explained variance, respectively, of the X and the Y matrices. $Q^2Y$ estimates the predictability of the model. $R^2 = 1$ indicates a perfect description of the data by the model whereas $Q^2 = 1$ indicates perfect predictability. For internal validation of the OPLS models we performed a permutation test (999 permutations). The aim of this test was to evaluate whether our OPLS models, built with the groups, was significantly better than any other OPLS model obtained by randomly permuting the original groups attribution.

For validation, a receiver–operator characteristic (ROC) curve was drawn to evaluate the ability of the OPLS latent variable $T_{pred}$ to correctly classify the test set. The area under the ROC (AUROC) was calculated. A perfect discrimination corresponded to an AUROC equal to 1. An optimal cut-off value of $T_{pred}$ ($T_{opt}$) was also calculated with the Matlab® function.
“percurve” which minimize both the false positive and false negative cases in the test set, assuming equal weightings on the cost of misclassification.

The small HCC set was used to test the ability of the model to discriminate between small HCC observations and cirrhosis cases. False positive and false negative rates were calculated using the curve cut-off, $T_{opt}$, previously calculated for the test set.

A score plot illustrated the results. Each point in the score plot represented the projection of an NMR spectrum (and thus a patient’s sample) on the predictive (horizontal axis) and the first orthogonal component of the model (vertical axis).

The loading plot represents the covariance between the Y-response matrix and the signal intensity of the various spectral domains. Colours were also used in the loading plot depending of the $p$-value associated with the correlation between the corresponding bucket intensity and the Y variable. The null hypothesis associated with the $p$-values used in this study is that there is no correlation between X (intensity of the buckets) and Y (cirrhotic group or HCC group) variables. To minimize false-positive rates in multiple comparisons of the 4239 spectral domains, we used the conservative Bonferroni correction, which discards any low significant variables. Thus, for an error rate of 0.01, a metabolite variation in the loading plot was considered significant if its $p$-value was $< 2.4 \times 10^{-6}$.

PCA and OPLS analyses were performed using Simca-P12 (Umetrics, Umea) and in-house Matlab® (Mathworks, Natick, MA) code based on Trygg and Wold method (14).

**Metabolite identification**

On the loading plot, the positive signals corresponded to those metabolites that had an increased concentration in the serum of patients with HCC. Conversely, a negative signal corresponded to those metabolites that had an increased concentration in the serum of patients without HCC.
The buckets were designated according to their central chemical-shift values. Their most probable assignment to a metabolite was given according to the spectral assignment, as previously described in the literature (15), and was confirmed with $^1$H-$^1$H TOCSY 2D NMR spectra.
RESULTS

Patients’ characteristics

A total of 286 patients were screened: of these, 158 patients fulfilled the inclusion criteria as shown in Figure 1. The data obtained by bucketing of the 158 serum spectra were successfully acquired and first analysed by PCA (results not shown). From this analysis, four outliers were identified. All spectra that exhibited high levels of ethanol (1.11 ppm and 3.66 ppm) were excluded. Demographics, clinical and biological features, as well as data regarding characteristics of liver tumours for the 154 remaining patients, are displayed in Table 1. As expected, patients with HCC were older and were more often male than patients without HCC. There was no difference in the severity of liver disease as assessed by the MELD score between the three groups.

OPLS model with cirrhosis and large HCC

Serum spectra from cirrhotic and large HCC groups that belonged to the training set were discriminated with the OPLS model as shown in the score plot in Figure 2a. The model was build with one predictive and 3 Y-orthogonal components and exhibited a good explained variance: ($R^2_X$) of 0.46, ($R^2_Y$) of 0.90, predictability ($Q^2_Y$) of 0.83.

The model was internally validated, as all $Q^2_Y$ and $R^2_Y$ values obtained with permuted Y were smaller than those of the model. Intercept values for $Q^2_Y$ and $R^2_Y$ obtained from the permutation plot (not shown) were, respectively, −0.57 and 1.40.

Validation performed with the test set shows that the model could predict cirrhosis or HCC as shown in the score plot in Figure 3a. In all cases, each sample was correctly classified according to the presence or absence of large HCC. Consequently, the AUROC was equal to 1 (not shown). The optimal cut-off value of Tpred was $T_{opt}=0.25$. 


Small HCC set

As shown in the score plot in Figure 3b the projection of cirrhotic and small HCC on the predictive component was heterogeneous.

Using the optimal cut-off value of $T_{\text{opt}}=0.25$, all cirrhotic sera were correctly assigned to the cirrhotic groups but, among the 28 small HCC group, 11 patients displayed a serum metabolic profile similar to that of patients with large HCC (true positive) while 17 had a metabolic profile close to that observed in cirrhotic patients without HCC (false negative).

Having identified two different metabolomic profiles in this subset of patients, we compared their baseline features as well as their outcomes after the RFA procedure (Table 2). If their clinical and radiological characteristics were comparable, there was a non significant trend towards higher rates of local or distant recurrence, as well as a higher rate of deaths during follow-up in patients with serum metabolomic profiles defining the true positive subset.

Discriminant metabolites

The appearance of $^1$H NMR serum spectra was globally similar to those previously described in human sera of cirrhotic patients (Supplementary Figure S1) (11). The spectral assignment was confirmed with $^1$H-$^1$H TOCSY 2D NMR spectra (Supplementary Figure S2).

The loading plot obtained with the OPLS model for the training set revealed that twelve signals, corresponding to eight metabolites or groups of metabolites, were highly significantly correlated with the presence of large HCC (Figure 2b). They are presented in Table 3.

In $^1$H NMR serum spectra, lipids could be detected as broad resonances of fatty-acid methyl and methylene moieties at 0.8 and 1.24 ppm, respectively, and the N-trimethyl moiety of choline was included in phospholipids at 3.22 ppm as shown in figure 1. Among those
signals, those at 0.8 and 1.24 ppm were significantly correlated to the presence of large HCC. These resonances of fatty acids arise from lipids included lipoprotein particles of different densities. It has been widely reported that high-density lipid (HDL) particles produce resonances with lower chemical shifts than low-density lipoprotein (LDL and VLDL) particles. Interestingly, lipoproteins with higher density were significantly elevated in cirrhotic patients without HCC. For VLDL, corresponding to the highest chemical shifts in methyl and methylene resonances, no differences were found. Others lipids were identified and participated in the discrimination.

Three other broad lipid signals were identified in the patients’ sera at 1.50, 1.99 and 5.33 ppm. The two first corresponded to methylene moieties following the ester moiety. The last one corresponded to methylene moiety following a double bound in unsaturated fatty acid.

N-acetyl moieties of glycoprotein produced a broad resonance at 2.05 ppm, which was increased for patients with HCC when compared to those without HCC. This signal corresponded to different N-acetylated glycoproteins as well as to their metabolites: N-acetylneuraminic acid and N-acetylglucosamine.

Some amino acids were identified in the spectra and were highly significant for discrimination. Glutamine was significantly higher in cirrhotic patients than HCC patients, whereas glutamate was significantly lower.

Ketone bodies could be detected in the spectra and participated in discrimination. Acetate (1.92 ppm) was significantly higher in HCC than cirrhotic patients.
Discussion

This preliminary study was conducted in a homogeneous and large sample-sized cohort of patients. The usual major limitation encountered in a translational metabolomic approach to liver diseases is methodological bias when selecting patients, thus limiting the confidence that can be drawn from conclusions (8, 9, 16). We attempted to avoid these pitfalls by constructing well-defined subgroups of patients according to their stage of underlying liver disease and HCC status. We also focused specifically on alcoholic aetiology to avoid any potential specific influence of viral or non-alcoholic-related liver disease on sera metabolomic profiles, as previously reported (17, 18). Moreover, to eliminate the effect of liver-function impairment on different metabolic pathways (as previously reported), we excluded all patients with end-stage liver disease from this analysis (11). Also, the collection of sera samples before the onset of any therapeutic procedure for HCC reinforced the confidence of the observed effect of tumour metabolism.

In this setting, the present data enabled us to draw several conclusions. First, it appears that changes in serum metabolomic profiles were observed according to the presence of advanced HCC. Second, these fingerprints reflected the distinct activation or impairment of multiple biological pathways, mainly energetic metabolism involving glutamine/glutamate, ketone bodies and lipids. On another hand, glucose sera content was not a discriminant metabolite. Lastly, the refinement of our initial projection, to analyse metabolic changes observed in biopsy-proven small-HCC patients, revealed various profiles that probably reflect different metabolic activities with potential clinical implications.

The metabolic profiles of cirrhotic patients with large HCC exhibited clear modifications in lipid composition, glutamine metabolism and ketone bodies compared to the metabolic profiles obtained from cirrhotic patients without HCC as previously described using mass spectroscopy or $^1$H NMR spectroscopy in urine, tissue or serum (7-10, 16, 19, 20). However,
those metabolic profiles were mainly described with viral etiology and not in alcoholic cirrhotic patients with well classified HCC.

Patients with advanced HCC had lower HDL than cirrhotic patients without HCC. Using serum $^1$H NMR spectroscopy of cirrhotic patients, we have previously confirmed that HDL level is altered and decreased with the severity of chronic liver failure (11). Patients with end-stage liver disease were excluded, thus, differences in HDL could not be explained by the severity of liver disease. Interestingly, several epidemiological studies have reported a good correlation between with low HDL-cholesterol levels and several cancers, including HCC (21). In the population studied here, cholesterolemia was not modifying in the presence of HCC (Table 1). Nevertheless, the lipoprotein profile, as assess with NMR spectra, showed that HDL fraction (not HDL-cholesterol) could be modulated in sera of patients with HCC. However, low HDL levels may be a non-specific surrogate marker for an existing malignancy rather than a predisposing factor. Unsaturated lipids were also found at higher levels in patients without cancer.

Glutaminolysis and glycolysis represent the main metabolic energy pathways in cell tumours. Alterations in glutamine metabolism in this context may have several explanations. First, ‘trapping’ of glutamine by malignant cells could explain the glutamine depletion in the serum of HCC patients when compared to the cirrhotic patients in our study. Yang et al showed, using HR MAS, in simultaneous samples of HCC and non-HCC tissues obtained from the same patients, elevated levels of glutamine in tumour samples: the levels were higher in high-grade tumours than low-grade or non-tumoral tissues (20). Glutamine consumption by tumour cells has been reported and seems to depend upon the presence or absence of an alternative energy substrate (22). Moreover, low levels of glutamine and high levels of glutamate in serum may be found in different states as cachexia, cancer (specifically digestive cancers) or pro-inflammatory states. In these clinical settings, high glutamate levels were secondary to the
accumulation of this metabolite in the serum in parallel with a decrease in muscular glutamate intake (23).

The role of glycoproteins in cancer disease has been widely studied. Using data from proton NMR spectroscopy of serum or plasma, the prominent resonance in the region of 2.0 to 2.1 ppm has been frequently associated with tumour invasion or malignancy in several types of cancer, including colorectal, gastric, breast, cervical, prostate and ovarian (6). Nicholson et al. have suggested that this resonance arises mainly from N-acetyl protons from alpha1 acid glycoprotein. This glycoprotein contains a high ratio of carbohydrate (45%), as N-linked glycans of a sialic-acid type (15). More specifically, in HCC, raised levels of alpha1 acid glycoprotein had been found in patients with cancer, as well as low AFP, when compared to cirrhotic patients without cancer (24).

Thus, serum metabolomic analysis of cirrhotic patients with large HCC highlights the activation of various metabolic pathways that seem to be specific to this tumour. Based on the present data, metabolomic NMR analysis provides new insights into the metabolic processes related to the biology of liver cancer. Furthermore, the projection of small HCC serum samples along pre-defined discriminant axes reveals important clinical information. Indeed, based on the serum analysis of these ‘intermediate’ patients, we were able to observe a subgroup of patients whose metabolomic profiles were similar to those of patients with large HCC, whereas other ‘intermediate’ patients displayed fingerprints suggesting an absence of liver cancer.

The model failed to clearly discriminate between cirrhotic patients and small HCC patients. However, from a metabolomic point of view, some patients with small HCC, who were eligible for curative treatment, seemed to behave as patients with advanced cancerous disease. HCC recurrence after curative treatment (whether RFA, surgical resection or transplantation) is a major challenge as tumour recurrence can be as high as 50% at 2 years (1). Thus, we
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attempted to classify small HCC patients according to their serum metabolomic profile and compared HCC characteristics within this subgroup (Table 2). We did not observe any differences according to clinical characteristics or HCC status, (number, size, histological features comprising expression of glutamine synthase): this suggests a certain degree of homogeneity that does not account for the identified metabolomic changes. However, there was a trend towards a worse prognosis in patients with profiles similar to the large HCC group, with higher rates of local or distant recurrences and a greater incidence of death, though these findings must be cautiously interpreted due to the small sample-size of this sub-population (25). By providing new patterns of recognition, metabolomic profiling may be a useful tool to refine prognosis and to aid therapeutic procedures for these patients.

However, metabolomic profiling, which assesses surrogate markers of tumour biology in tumoural tissues or serum samples, can provide complete and multivariate information on metabolomic changes related to liver tumours and can possibly assess its aggressiveness, thus defining various metabolic profiles. The assessment of this technique as a prognosis tool in patients with HCC and who are eligible for curative treatment deserves further investigation, particularly in HCV- or HBV- infected HCC patients in whom metabolomic changes reported here may not be entirely translated. Most importantly, the intra-individual analysis of serum changes during follow-up of treated patients may provide essential information related to therapeutic responses, recurrence or progression. These new approaches should be developed in large prospective cohorts of well-defined patients undergoing standardized therapeutic procedures classified according to both the etiology on the underlying liver disease and the stage of liver tumour, and in whom sequential biological samples are available for metabolomic profiling. Finally, the additional integration of metabolomic information, which may improve the performance of pre-existing risk-assessment models for HCC occurrence or recurrence after treatment, warrants to be tested in large cohorts of prospective followed-up
patients with cirrhosis. Such approach will surely improve our understanding of the implications of the various biological pathways involved in the progression towards cancer diseases and may, in the near future, help identify subgroups of patients at high risk of HCC, and so could benefit from specific preventive measures or could be given adapted screening policies.
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References


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Table 1

<table>
<thead>
<tr>
<th></th>
<th>Training set (n=84)</th>
<th>Test set (n=42)</th>
<th>Small HCC Projections (n=28)</th>
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<tbody>
<tr>
<td></td>
<td>Cirrhosis (n=62)</td>
<td>Large HCC (n=22)</td>
<td>Cirrhosis (n=31)</td>
</tr>
<tr>
<td>Age (years)</td>
<td>59.0±1.4</td>
<td>71.7±1.7*</td>
<td>59.5±2.2</td>
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<tr>
<td>Male gender</td>
<td>53 (85%)</td>
<td>22 (100%)*</td>
<td>27 (81%)</td>
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<tr>
<td>ALT (UI/L)</td>
<td>41.1±2.5</td>
<td>53±11.4</td>
<td>47.0±7.8</td>
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<td>AST (UI/L)</td>
<td>75.9±6.8</td>
<td>97.3±26.7</td>
<td>72.4±12.4</td>
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<td>Albumin (g/L)</td>
<td>38.9±0.7</td>
<td>37.3±1.0</td>
<td>37.9±1.2</td>
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<td>Prothrombin level (% control)</td>
<td>72.5±2.2</td>
<td>75.4±3.7</td>
<td>70.0±3.5</td>
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<td>Bilirubin (µmol/L)</td>
<td>23.6±3.3</td>
<td>17.9±2.1</td>
<td>29.4±4.1</td>
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<td>Child Pugh score a</td>
<td>5.8±0.1</td>
<td>6.0±0.2</td>
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<td>MELD score a</td>
<td>11.3±0.5</td>
<td>11.1±1.0</td>
<td>11.9±0.8</td>
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<td>Creatinine (µmol/L)</td>
<td>87.0±4.8</td>
<td>101.3±8.6</td>
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<td>Blood Glucose (mmol/L)</td>
<td>6.3±0.3</td>
<td>6.1±0.3</td>
<td>8.0±0.9</td>
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<td>Triglyceride (g/L) a</td>
<td>1.1±0.1</td>
<td>1.5±0.3</td>
<td>1.5±0.2</td>
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<td>Total cholesterol (mmol/L) a</td>
<td>4.4±0.2</td>
<td>4.9±0.6</td>
<td>4.6±0.3</td>
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<td>AFP (ng/mL) a</td>
<td>10.3±3.1</td>
<td>6156.2±3947.6*</td>
<td>18.0±4.0</td>
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HCC Characteristics

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<table>
<thead>
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<tr>
<td>Number of nodules</td>
<td>2.4±0.3</td>
<td>3.6±0.8</td>
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<tr>
<td>Size (mean sum of nodules, mm)</td>
<td>93.1±9.4</td>
<td>100.1±12.2</td>
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<tr>
<td>PVT (number, %)</td>
<td>1 (2%)</td>
<td>8 (36%)</td>
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<tr>
<td>Metastasis (number, %)</td>
<td>3 (14%)</td>
<td>1 (9%)</td>
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### Table 2

<table>
<thead>
<tr>
<th></th>
<th>False Negative(^c)</th>
<th>True Positive(^d)</th>
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<tbody>
<tr>
<td><strong>Age (years)</strong> (^a)</td>
<td>64.8±1.7</td>
<td>69.9±1.8</td>
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<tr>
<td><strong>Male gender</strong> (^b)</td>
<td>12/17</td>
<td>10/11</td>
</tr>
<tr>
<td><strong>Child</strong></td>
<td>5.2±0.1</td>
<td>6.1±0.3</td>
</tr>
<tr>
<td><strong>MELD</strong></td>
<td>9.9±0.5</td>
<td>11.5±1.2</td>
</tr>
<tr>
<td><strong>Number of Tumors</strong></td>
<td>1.3±0.1</td>
<td>1.2±0.1</td>
</tr>
<tr>
<td><strong>Size of Tumors (mm)</strong></td>
<td>33.2±4.5</td>
<td>30.1±3.1</td>
</tr>
<tr>
<td><strong>Edmondson grade (I/II/III)</strong></td>
<td>3/11/3</td>
<td>2/7/2</td>
</tr>
<tr>
<td><strong>Liver Glutamine synthase</strong> (^e)</td>
<td>38%</td>
<td>25%</td>
</tr>
<tr>
<td><strong>Recurrence</strong> (^b)</td>
<td>7 (41%)</td>
<td>7 (63%)</td>
</tr>
<tr>
<td><strong>Overall Mortality</strong> (^b)</td>
<td>4 (23%)</td>
<td>6 (54%)</td>
</tr>
</tbody>
</table>
### Table 3

<table>
<thead>
<tr>
<th>Number</th>
<th>Name</th>
<th>Chemical shift (ppm) and multiplicity</th>
<th>$r$</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Methyl moieties of fatty acids (HDL)</td>
<td>0.84$^m$ 1.23$^n$</td>
<td>-0.60</td>
<td>5.22 $10^{-9}$</td>
</tr>
<tr>
<td>2</td>
<td>Methylene moieties of fatty acids</td>
<td>1.50$^m$</td>
<td>-0.56</td>
<td>4.09 $10^{-6}$</td>
</tr>
<tr>
<td>3</td>
<td>Acetate</td>
<td>1.90$^s$</td>
<td>+0.57</td>
<td>2.56 $10^{-8}$</td>
</tr>
<tr>
<td>4</td>
<td>Methylene moieties of fatty acids ($\beta$ of esters)</td>
<td>1.99$^b$</td>
<td>-0.48</td>
<td>5.42 $10^{-6}$</td>
</tr>
<tr>
<td>5</td>
<td>N-acetyl moiety</td>
<td>2.02$^m$</td>
<td>+0.52</td>
<td>5.68 $10^{-7}$</td>
</tr>
<tr>
<td>6</td>
<td>Glutamate</td>
<td>2.33$^m$</td>
<td>+0.83</td>
<td>1.23 $10^{-21}$</td>
</tr>
<tr>
<td>7</td>
<td>Glutamine</td>
<td>2.11$^m$ 2.43$^n$</td>
<td>-0.63</td>
<td>3.92 $10^{-12}$</td>
</tr>
<tr>
<td>8</td>
<td>Fatty acids double bounds</td>
<td>5.33$^b$</td>
<td>-0.42</td>
<td>6.50 $10^{-7}$</td>
</tr>
</tbody>
</table>
Table legends

Table 1: Baseline characteristics of the population
All biological and clinical parameters were recorded at inclusion. \(^{a}\)Mean ± SEM. \(^{b}\)Number (percentage) of patients. HCC: hepatocarcinoma

*P<0.05 between HCC and Cirrhosis
\(^{\mu}\) P<0.05 between Large and Small HCC Groups

Table 2: Characteristics of small HCC according to their projection on the model
\(^{a}\)Mean ± SEM. \(^{b}\)Number (percentage) of patients. \(^{c}\)False negative corresponds to proven-biopsy HCC case projected with the cirrhosis cases. \(^{d}\)True positive corresponds to proven-biopsy HCC case projected with Large HCC cases. \(^{e}\)Glutamine synthase was considered positive when more than 50% of tumor cells showed a strong cytoplasmic staining. Results were expressed in percentage of positive biopsy

Table 3: Discriminant metabolites observed by \(^1\)H NMR spectroscopy, according to the loading plot (Figure 2b), between cirrhotic serum spectra and Large HCC serum spectra.
The metabolite number corresponds to the label in the loading plot in Figure 2b. Chemical shift (referenced to fumaric acid signal at 6.53 ppm) and multiplicity correspond to those found in the \(^1\)H NMR spectra of the patient’s serum at 298K. ppm: part per million, \(^{\circ}\)singlet, \(^{\dd}\)doublet, \(^{\text{dd}}\)doublet of doublet, \(^{\text{t}}\)triplet, \(^{\text{m}}\)multiplet, \(^{\text{b}}\)broad signal. A negative correlation (\(r\)) corresponds of an increased metabolite concentration in the cirrhotic group and a positive correlation to an increased metabolite concentration in the HCC group.
Figure legends

Figure 1: Flow chart of patients.
HCC: Hepatocellular carcinoma; CPT: Child–Pugh–Turcott score; AoCLF: acute on chronic liver failure; PCA: principal component analysis; OPLS: orthogonal partial latent structure analysis

Figure 2: OPLS score plot (a) and Loading plot (b).
(a): On the score plot, each dot corresponds to a spectrum coloured according to the absence (blue) or the presence (red) of hepatocellular carcinoma (HCC). The constructed model displays a good separation between the spectrum of cirrhotic patients without HCC and those with HCC.
(b): On the Loading plot, variations of bucket intensities are represented using a line plot between 0 to 6 ppm. Positive signals correspond to the metabolites present at increased concentrations in patients with large HCC. Conversely, negative signals correspond to the metabolites present at increased concentrations in patients without HCC. The buckets are labelled according to the metabolite as presented in Table 2.

Figure 3: Validation of the model (a) and projection of serum spectra in patients from the cirrhosis or small HCC group (b).
Each dot corresponds to a spectrum coloured depending on the absence (blue) or presence (red) of hepatocellular carcinoma (HCC).
(a): Each new spectrum was projected in the score plot using the previously constructed model to enable prediction of the presence or absence of HCC. The area under the a receiver–operator characteristic (AUROC) drawn to evaluate the ability of the OPLS (orthogonal
projection to latent structure analysis latent variable, $T_{\text{pred}}$, to correctly classify each spectrum was equal to 1 with a cut-off of $T_{\text{pred}} (T_{\text{opt}})$ equal to 0.25.

(b): Using the same previously calculated cut-off value of the training set ($T_{\text{opt}}=0.25$), each spectrum of cirrhotic patients without HCC was correctly assigned. Nevertheless, the spectra from small HCC patients had a heterogeneous distribution in the score plot. Some were projected on the left with the cirrhotic spectra, others were projected on the right, with the large HCC patients, and some were projected in between.
Figure 1

286 patients with biopsy proven cirrhosis (with or without HCC)

158 cirrhotic patients included and with acquired serum spectra

154 patients included for OPLS analysis

Cirrhosis without HCC = 93

Cirrhosis with HCC = 61

Large HCC = 33

Small HCC = 28

128 patients excluded for one or more criteria:
- 82 CPT score > 8
- 6 Viral infections
- 11 previously treated HCC
- 6 acute alcoholic hepatitis
- 18 Recent AoCLF (less than 6 months)
- 12 concomitant cancers

4 Outliers excluded due to ethanol pic after PCA
Figure 3

(a) 

T_{orth} vs. T_{pred}

- Blue circles: Cirrhotic patient without HCC
- Red circles: Cirrhotic patient with Large HCC

(b) 

T_{orth} vs. T_{pred}

- Blue circles: Cirrhotic patient without HCC
- Red circles: Cirrhotic patient with Small HCC
Identification of serum proton NMR metabolomic fingerprints associated with hepatocellular carcinoma in patients with alcoholic cirrhosis

Pierre Nahon, Roland Amathieu, Mohamed N. Triba, et al.

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