

Plasma Lipidomic Fingerprinting to Distinguish among Hepatitis C-related Hepatocellular Carcinoma, Liver Cirrhosis, and Chronic Hepatitis C using MALDI-TOF Mass Spectrometry: a Pilot Study

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ABSTRACT

Background & Aims: Hepatitis C (HC) is a major cause of hepatocellular carcinoma (HCC), and a late diagnosis is the main factor for the poor survival of patients. There is an urgent need for identifying sensitive and specific biomarkers for HCC diagnosis. In the present study, plasma lipid patterns of patients with HC-HCC, HC-liver cirrhosis (LC), and chronic HC (CHC) were assessed by matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS).

Methods. Plasma samples of 25 patients with HC-HCC, 15 patients with HC-LC, and 25 patients with CHC were evaluated by MALDI-MS using a Q-ToF premier (Synapt) mass spectrometer (Waters, Manchester, UK) equipped with a 200-Hz solid-state laser in the mass range between m/z (mass-to-charge ratio) of 700–1200.

Results. A total of 2205 ions were initially obtained and 7 ions (m/z) were highlighted as corresponding to the most important lipids to differentiate HCC patients from LC and CHC patients. The specific lipidomic expression signature generated resulted in an overall predictive accuracy of 93% of HC-HCC and HC-LC, and 100% of HC-HCC and CHC. The 7-peak algorithm distinguished HCC from LC with a sensitivity of 96% and a specificity of 87%, and HCC from CHC with both sensitivity and specificity of 100%.

Conclusion. MALDI-MS-specific signature peaks accurately distinguished patients with HC-HCC from those with HC-LC and CHC. The results indicate the potential of MALDI-MS and the selected peaks to improve HCC surveillance in patients with viral C cirrhosis and chronic hepatitis C.

Key words: hepatocellular carcinoma – hepatitis C – lipidomics – fingerprints – lipids – MALDI-MS.

Abbreviations: AFP: alpha-fetoprotein; AUC: area under the curve; CHC: chronic hepatitis C; HC: hepatitis C; HCC: hepatocellular carcinoma; HCV: hepatitis C virus; LC: liver cirrhosis; MALDI: matrix-assisted laser desorption/ionization; MeOH: methanol; MS: mass spectrometry; ROC: receiver operating characteristic; VIP: variable importance in the projection.

INTRODUCTION

Hepatitis C virus (HCV) infection is one of the main causes of chronic liver disease worldwide [1]. It is estimated that 160 million individuals are chronically infected with HCV [2]. Depending on the presence of co-factors, up to 40% of patients with chronic HCV infection will develop liver cirrhosis (LC) [3]. Death related to complications of LC occurs at

an incidence of approximately 4% per year worldwide, whereas hepatocellular carcinoma (HCC) occurs in this population at an estimated incidence of 1–5% per year [4-6]. In Brazil, HCV accounts for over half of the HCC cases, whereas LC is present in over 90% of HCC cases [7].

Hepatocellular carcinoma is a complex and heterogeneous tumor with several genomic alterations [8]. Despite significant progress in diagnosis and treatment options, the incidence of HCC is still increasing. When HCC is diagnosed at an early stage, surgical options such as resection or liver transplantation, or local ablative therapies can be applied with intent to cure. However, no effective serum or plasma biomarkers have been found or diagnosis procedures developed. Therefore, patients at risk for HCC should be enrolled in a surveillance program [9].

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Diagnosis of HCC is most commonly performed by ultrasound examination, while CT scan, magnetic resonance, and histopathology may also be used, despite some limitations related to risk of complications and feasibility of the biopsy due to tumor location. Imaging techniques have widespread use due to their non-invasiveness, safety, relatively moderate cost, and good acceptance by patients. However, the effectiveness of such techniques for early detection of HCC highly depends on the stage of liver fibrosis, the quality of the equipment, and the expertise of the operator [9].

Alpha-fetoprotein (AFP) is the most widely used biomarker for HCC. However, its sensitivity is only up to 60% and elevated AFP levels are also common in LC and chronic liver disease. Assessment of AFP levels has recently been removed from the American Association for the Study of Liver Diseases guidelines as a procedure for HCC diagnosis. Therefore, there is a need to identify better HCC biomarkers [10]. The ideal marker should be specific and able to discriminate HCC from regenerative nodules regardless of cause and stage of liver disease. Furthermore, the marker should be sensitive, allowing detection at an early stage, and should be easily measurable, reproducible, and minimally invasive.

Recently developed mass spectrometry (MS)-based techniques such as proteomics, lipidomics, and metabolomics represent promising tools for the discovery and subsequent identification of proteins, peptides, lipids, and metabolites associated with various diseases. For instance, the development of matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) has provided a powerful tool for discovery of biomarkers in different kinds of cancers [11-13]. However, studies on lipid profiling and fingerprinting of serum and/or plasma samples from HCCs are still scarce [14-16]. We assessed the plasma lipid patterns of patients having HC-HCC, HC-LC, and CHC by performing MALDI-MS, and identified some potential biomarkers that resulted in a lipid algorithm that may be used in HCC surveillance programs of HC-LC and CHC patients.

PATIENTS AND METHODS

Study design and sample collection

This study included 25 patients with HC-HCC, 15 patients with HC-LC, and 25 patients with CHC. Chronic hepatitis C was diagnosed based on the presence of antibodies specific to HCV and HCV RNA for over six months. Liver cirrhosis was diagnosed by histopathology. Hepatocellular carcinoma was diagnosed using imaging or histopathology, in accordance with the guidelines of the Brazilian Society of Hepatology. Patients were characterized according to age and gender. All patients in the HC-LC and CHC groups were followed for a minimum of 12 months to discard undiagnosed HCC. Blood samples were obtained by venipuncture and drained into blood-collection tubes with EDTA. Blood samples were centrifuged immediately after collection and plasma was stored in 500 μ L aliquots at -80°C until analysis.

The study was approved by the Ethics Committee of our institution and all patients gave written informed consent.

Chemicals and materials

All chemicals were of analytical reagent grade and used as received. Chloroform (CHCl_3) and methanol (MeOH) were purchased from Burdick & Jackson (Muskegon, MI, USA). 2,5-Dihydroxybenzoic acid (DHB) was purchased from ICN Biomedicals (Aurora, OH, USA). Deionized water was obtained from a Millipore Milli-Q water reagent system (Millipore, Bedford, MA, USA). EDTA tubes were purchased from BD Diagnostics (Franklin Lakes, NJ, USA).

Extraction of lipids

Lipids were extracted from each sample using the Blish-Dyer protocol [17]. Immediately after thawing, 50 μ L of plasma were dissolved in a mixture of chloroform-methanol (125:250 μ L) and vortexed well. After vortexing, 125 μ L of chloroform and 100 μ L of deionized water were added to supernatant and centrifuged at 1000 rpm in a tabletop centrifuge for 5 min at room temperature. Following this protocol a two-phase system (aqueous top, organic bottom) was achieved. The bottom phase containing lipids was gently recovered using a micropipette, dried, and sealed to be stored at -80°C until analysis.

Mass spectrometry analysis

The MALDI-MS method was applied for lipidomic fingerprinting assessment in plasma to identify differential lipidomic expression signatures and lipidomic profiles in patients with HC-HCC, HC-LC, and CHC. MALDI-MS spectra were acquired in the positive ion mode using a Q-ToF premier (Synapt) mass spectrometer (Waters, Manchester, UK) equipped with a 200-Hz solid-state laser in the mass range between m/z (mass-to-charge ratio) of 700-1200. The operating principle used was 10 V (sample plate), and laser irradiation consisted of several shots in the region where the sample had been placed on the target plate for 60-90 sec, until signals in the region of interest were observed and disappeared due to consumption of the microscopic sample. Spectra were centered and aligned using MassLynx 4.0 software (Waters, Manchester, UK). After attribution, only the m/z values that were clearly distinct from noise level in the spectra were included in the partial least square discriminant analysis (PLS-DA), which was performed using MetaboAnalyst 2.0 (The Metabolomics Innovation Centre, Canada) [18, 19].

Data processing

The data matrix was exported for PLS-DA. To find differential circulating lipids, a VIP (variable importance in the projection) parameter was employed to reflect the variable importance in the discriminant analysis. The major discriminant variables with VIP values > 2.0 were subjected to Student's t test ($P < 0.01$) and fold change analysis (> 2). Furthermore, significant variables were selected and compared using the Mann-Whitney U test to confirm the differential expression between groups. Peaks with $P < 0.01$ were considered statistically significant.

The receiver operating characteristic (ROC) curves were independently generated for each differentiating peak and the area under the curve (AUC) was calculated. Cut-off values for

each ion were determined based on the normalized abundance that yielded the higher efficiency (arithmetic average of sensitivity and specificity) in the ROC curve. Peaks were classified as “positive” if values were lower than or equal to the cutoff value. Otherwise, they were classified as “negative.” Each positive peak was assigned a score value of 1 and the total score per sample was assessed by a ROC curve, which determined the cutoff value of the peak-algorithm. Statistics were performed using SPSS version 11.0 (SPSS Inc., Chicago, IL, USA).

RESULTS

The mean age of the patients was 65 years (± 7 years, median 63 years) in the HC-HCC group, 47 years (± 9 years, median 49 years) in the HC-LC group, and 42 years (± 13 years, median 41 years) in the CHC group. Sixty per cent (15/25) of patients

in the HC-HCC group, 53% (8/15) in the HC-LC group, and 52% (13/25) in the CHC group were males.

Base peak chromatograms of plasma samples were created for the HC-HCC, HC-LC, and CHC groups. A total of 2205 ions were identified.

Figure 1 shows the PLS-DA score plot for the three groups evaluated. All variables with a VIP value > 2 were analyzed as to statistical significance in Student's *t* test ($P < 0.01$) and fold change (> 4) between groups. Selected variables were subjected to an independent statistical reevaluation using Mann-Whitney *U* test. Seven variables with a $P < 0.01$ were recognized as the most important lipids for the discrimination of patients with HC-HCC.

Hepatocellular carcinoma versus liver cirrhosis

Seven lipids independently predicted HC-HCC from LC with 76–80% accuracy, 88–100% sensitivity, and 53–60%

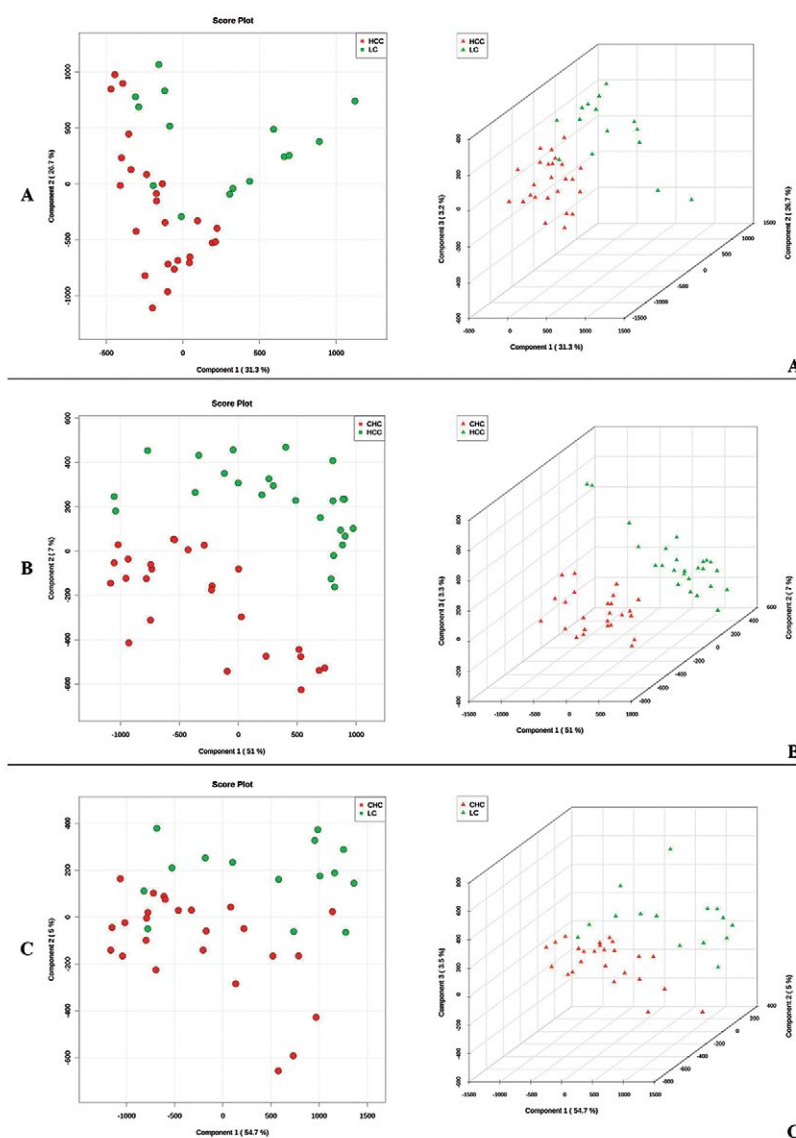


Fig. 1. 2D and 3D partial least squares discriminant analysis (PLS-DA) using MALDI-MS lipid data from plasma samples of patients with (A) hepatitis C-hepatocellular carcinoma and liver cirrhosis, (B) hepatitis C-hepatocellular carcinoma and chronic hepatitis C, and (C) liver cirrhosis and chronic hepatitis C.

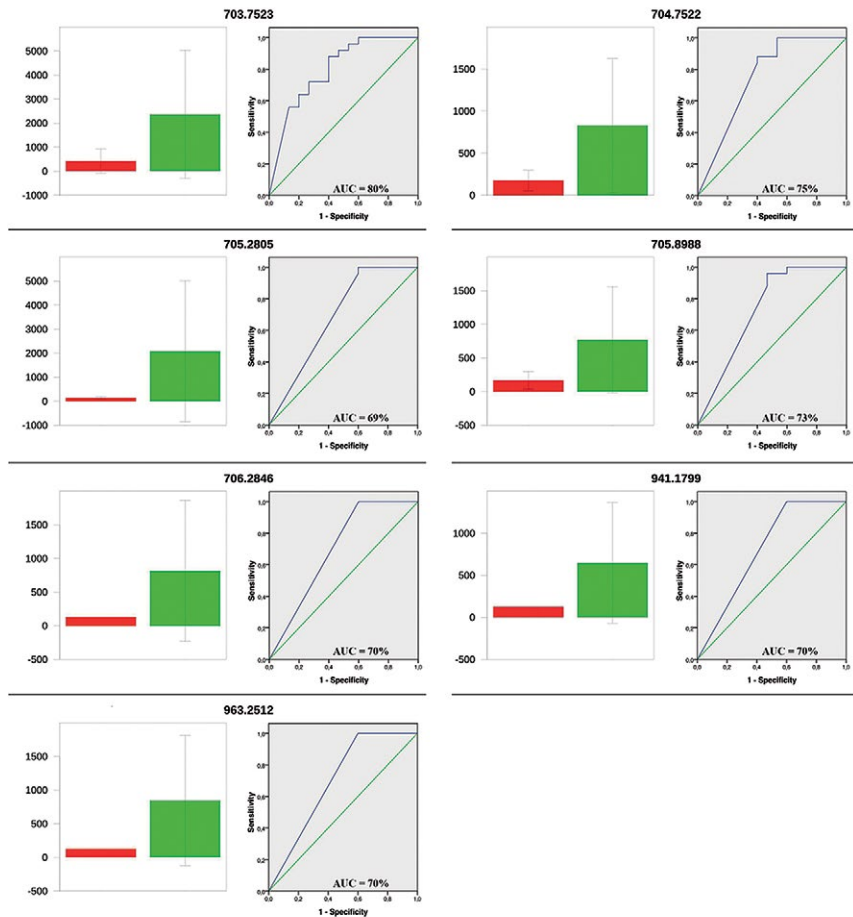


Fig. 2. Original intensities of differential ions by m/z in hepatitis C-related hepatocellular carcinoma (red boxes) and hepatitis C-related liver cirrhosis (green boxes). Receiver operating characteristic (ROC) curves calculated using cross-validated normalized values of partial least squares differential analysis (PLS-DA) model in discriminating the two groups. AUC, area under the curve.

specificity. Figure 2 shows the intensities and ROC curves of the seven lipids in patients with HC-HCC and HC-LC.

Based on the efficiency of each ROC curve, cutoff values were determined for each ion. The number of “positive” ions in each sample was used to generate a seven-peak algorithm with cutoff value of at least five “positive” biomarkers, defined by ROC curve analysis (Fig. 3A). The seven-peak algorithm generated distinguished HC-HCC from HC-LC with an accuracy of 93% (95% CI: 80–97%), a sensitivity of 96% (95% CI: 80–99%), and a specificity of 87% (95% CI: 62–96%).

This algorithm successfully detected 24 of 25 HC-HCC cases when applied to differentiate HCC from LC. Conversely, AFP detected only 7 of 25 HC-HCC cases from LC when cutoff value was set as 200 ng/mL, showing an accuracy of 55% (95% CI: 40–70%), a sensitivity of 28% (95% CI: 14–48%), and a specificity of 100% (95% CI: 80–100%). In the range of 20 ng/mL, AFP detected 18 of 25 HC-HCC cases, performing with an accuracy of 70% (95% CI: 55–82%), a sensitivity of 72% (95% CI: 52–86%), and a specificity of 67% (95% CI: 42–85%) (Fig. 3C).

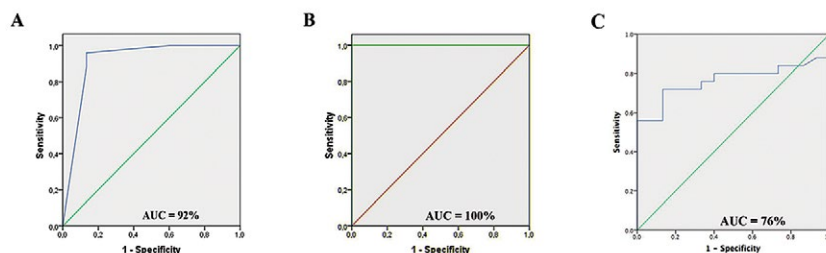


Fig. 3. Receiver operating characteristic (ROC) curves calculated using scores of “positive” ions for each sample in the (A) HC-HCC and HC-LC groups, (B) HC-HCC and CHC groups, and (C) using AFP values as ng/mL in the HC-HCC and HC-LC groups. AUC, area under the curve.

Hepatocellular carcinoma versus chronic hepatitis C

Seven lipids independently predicted HC-HCC from CHC with 68–94% accuracy, 96–100% sensitivity, and 40–88% specificity. Fig. 4 shows the intensities and ROC curves of the seven lipids in patients with HC-HCC and CHC.

The seven-peak algorithm generated (Fig. 3B) distinguished HC-HCC from CHC with an accuracy of 100% (95% CI: 93–100%), a sensitivity of 100% (95% CI: 87–100%), and a specificity of 100% (95% CI: 87–100%). All HC-HCC cases were successfully detected by this algorithm when applied to differentiate HCC from CHC.

Identification of potential biomarkers

We performed a tentative identification of the differentiating lipids on the LIPID MAPS database (<http://www.lipidmaps.org>), using the results of m/z analyses. Table I shows the main classes and subclasses associated with each m/z result of the seven different lipids found in HC-HCC samples.

DISCUSSION

In this study, a MALDI-MS based plasma lipidomic expression signature successfully detected 24 of 25 HC-HCC cases when applied to distinguish HCC and LC and provided a more precise diagnosis instrument for cirrhotic patients

than conventional non-invasive biomarker detection. Our results also show that MALDI-MS lipidomic fingerprinting discriminated plasma lipidomic expression patterns among patients with HC-HCC, HC-LC, and CHC.

Alpha-fetoprotein is known to misdiagnose up to 40% of HCC cases when the cut-off value is set at 20 ng/mL [20]. In this study, our diagnostic model accurately diagnosed 24 of 25 HCC cases from HC-LC patients, with a sensitivity of 96% and a specificity of 87%, while AFP performed poorly, diagnosing only 7 of 25 HCC cases when the cutoff value was 200 ng/mL and 18 of 25 HCC cases when cutoff was set as 20 ng/mL. Therefore, we propose this model to be applied in HC-LC patients for HCC surveillance.

Interestingly, all lipids identified in the model were down-regulated in the HC-HCC group when compared to the HC-LC and CHC groups. HCV replication can result in the disruption of several aspects of lipid metabolism in infected hepatocytes, as it enhances its replication by modulating host cell lipid metabolism, negatively modulates the synthesis and secretion of very low-density lipoproteins, and circulates in the blood in association with lipoproteins [21]. However, there is limited information available on the impact of HCV infection on global metabolism [22].

A total of 2205 signals were identified in our study. The main classes of lipids down-regulated in the HC-HCC

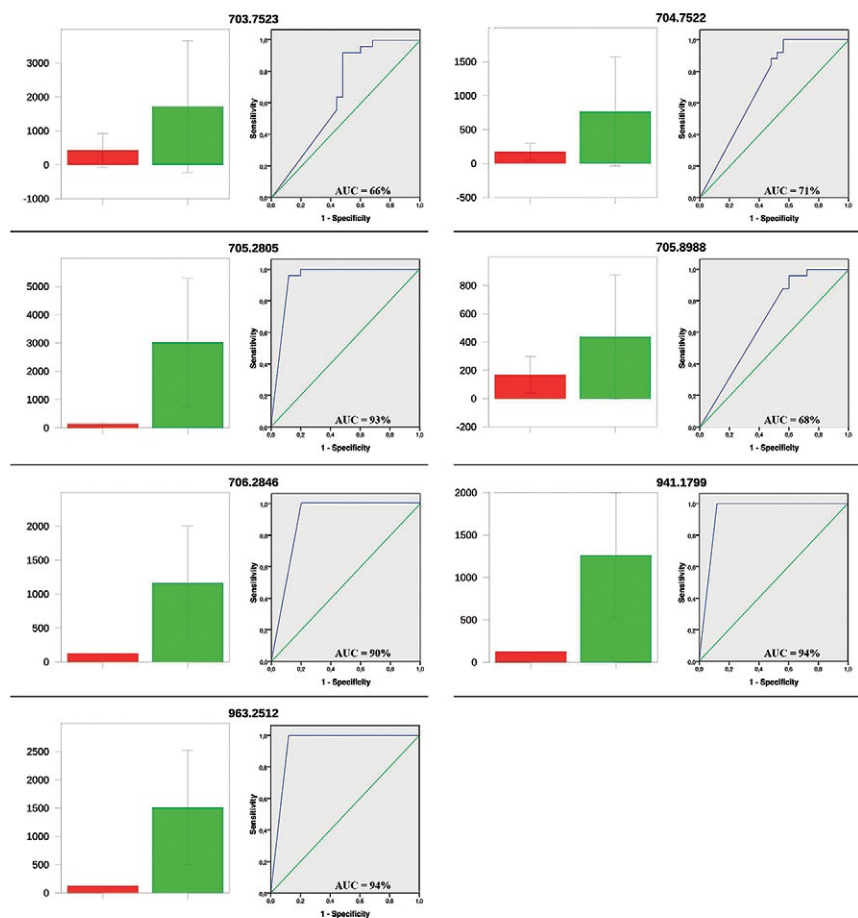


Fig. 4. Original intensities of differential ions by m/z in hepatitis C-related hepatocellular carcinoma (red boxes) and chronic hepatitis C (green boxes). Receiver operating characteristic (ROC) curves calculated using cross-validated normalized values of partial least squares differential analysis (PLS-DA) model in discriminating the two groups. AUC, area under the curve.

Table I. Main classes and sub classes of the differential lipids in hepatitis C related hepatocellular carcinoma (HC-HCC).

m/z	Main class	Formula	Sub class
703.7523	Sterols [ST01]	C ₄₉ H ₈₃ O ₂	Cholesteryl esters [ST0102]
704.7522	Glycerophosphocholines [GP01]	C ₃₉ H ₇₉ NO ₇ P	1-alkyl,2-acylglycerophosphocholines [GP0102]
705.2805	Glycerophosphates [GP10]	C ₄₀ H ₆₆ O ₈ P	Diacylglycerophosphates [GP1001]
705.8988	Fatty Acids and Conjugates [FA01]	C ₄₈ H ₉₇ O ₂	Branched fatty acids [FA0102]
706.2846	Glycerophosphoserines [GP03]	C ₃₆ H ₆₉ NO ₁₀ P	Diacylglycerophosphoserines [GP0301]
941.1799	Glycerophosphoinositols [GP06]	C ₅₁ H ₉₀ O ₁₃ P	Diacylglycerophosphoinositols [GP0601]
963.2512	Glycerophosphoinositols [GP06]	C ₅₃ H ₈₈ O ₁₃ P	Diacylglycerophosphoinositols [GP0601]

group were sterols [ST01], glycerophosphocholines [GP01], glycerophosphates [GP10], fatty acids and conjugates [FA01], glycerophosphoserines [GP03], and glycerophosphoinositols [GP06] (Table I).

CONCLUSIONS

Our findings suggest that MALDI-MS lipidomic fingerprinting may be a powerful tool for the identification of diagnostic biomarkers and models for HC-HCC. We demonstrated that the lipid fingerprinting in HC-HCC patients selected a number of lipids that should be functionally investigated to elucidate the pathogenesis of the disease. Furthermore, our results indicate the potential of this technique and the selected peaks to improve HCC surveillance in patients with viral C cirrhosis and chronic hepatitis C.

Conflicts of interest: No conflict to declare.

Authors' contribution: A.M.P.-C., C.G.: study conception and design and drafting of the manuscript. E.L.T., M.L.F., C.M., I.S., E.Parise, E.Pilau, F.G.: acquisition of data and critical revision. A.M.P.-C., E.L.T., E. Pilau, C.G.: analysis and interpretation of data. All authors approved the final version of the manuscript.

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