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Name of Journal: *World Journal of Hepatology*

Manuscript NO: 87666

Manuscript Type: ORIGINAL ARTICLE

Basic Study

Metabolomics in Chronic Hepatitis C: Decoding Fibrosis Grading and Underlying Pathways

Metabolomics in CHC: Fibrosis Grading

Abstract

BACKGROUND

Chronic Hepatitis C (CHC) affects 71 million globally, leading to liver issues like fibrosis, cirrhosis, cancer, and death. Better understanding and prognosis of liver involvement are vital to reduce morbidity and mortality. Accurate fibrosis stage identification is crucial for treatment decisions and predicting outcomes. Tests used to grading fibrosis include histological analysis and imaging but have limitations. Blood markers, like molecular biomarkers, can offer valuable fibrosis insights.

AIM

Analyze the plasmatic metabolome of CHC patients, looking for potential biomarkers to stratify these lesions and adding information about the molecular mechanisms involved in the disease.

METHODS

Plasma samples were collected from 46 patients with hepatitis C and classified into fibrosis grade: F1 ($n = 13$), F2 ($n = 12$), F3 ($n = 6$), and F4 ($n = 15$). To ensure that the biomarkers found are exclusive to liver lesions (chronic hepatitis C - fibrosis), healthy volunteer participants (healthy control group; $n = 50$) were included in the study. An

untargeted metabolomic technique was used to analyze the plasma metabolites by using mass spectrometry along with database verification. Statistical analyses were performed to identify differential biomarkers among the groups.

RESULTS

Six differential metabolites were identified for each fibrosis grade. The highlighted metabolites identified were able to establish an interesting clustering tendency of patients with the same grade of fibrosis, thus, showed more efficiency in discriminating the grades.

CONCLUSION

In conclusion, these results suggest that some of the observed biomarkers, once validated, have the potential for application as prognostic biomarkers. In addition, this study suggests that liquid biopsy analyzes of plasma metabolites are a good source of molecular biomarkers capable of stratifying patients with chronic hepatitis C according to their fibrosis grade.

Key Words: Chronic Hepatitis C; Fibrosis; Metabolome; Biomarkers; Plasma; Liquid biopsy

Ferrasi AC, Lima SVG, Galvani AF, Delafiori J, Dias-Audibert FL, Catharino RR, Silva GF, Praxedes RR, Santos DB, Almeida DTM, Lima EO. Metabolomics in Chronic Hepatitis C: Decoding Fibrosis Grading and Underlying Pathways. *World J Hepatol* 2023; In press

Core Tip: Chronic Hepatitis C (CHC) affects 71 million globally, leading to liver issues like fibrosis, cirrhosis, cancer, and death. Thus, accurate fibrosis stage identification is crucial for treatment decisions and predicting outcomes. Blood markers are a relevant source of information and different molecular biomarkers have been investigated for

the characterization of liver fibrosis. Here, we analyze the plasma metabolites by using mass spectrometry of 46 patients with hepatitis C and classified them into fibrosis grades (F1-F4) and 50 healthy volunteer participants (Healthy Control Group - CG). We identified six differential metabolites for each fibrosis grade that were able to discriminate it. We also analyzed the pathways linked to the main metabolites detected, adding information about the molecular mechanisms involved in the disease.

1

INTRODUCTION

Chronic hepatitis C (CHC) is an infectious disease caused by the hepatitis C virus (HCV) and is a serious public health problem, affecting an estimated 71 million people worldwide^[1-3].

3

Approximately 50-80% of HCV-infected individuals develop chronic hepatitis C, which can trigger a chronic inflammatory disease process leading to liver fibrosis, cirrhosis, hepatocellular carcinoma (HCC), and death^[4].

The natural progression of chronic HCV infection occurs with sustained inflammation due to repetitive liver injury followed by activation of hepatic stellate cells (HSC), deposition of fibrillar collagen in the extracellular matrix (ECM), and progressive fibrosis^[5,6]. These progressive processes may result in ECM degradation and, consequently, in vascular and architectural alterations, leading to a worse situation, cirrhosis (10%-20% of patients)^[7] and hepatocellular carcinoma (HCC) (1%-5%)^[8].

Early diagnosis and treatment can prevent liver cirrhosis and HCC, especially with the application of screening and recent advances in CHC treatment based on direct-acting antiviral (DAA) therapy. However, effective reduction of disease morbidity and mortality requires better characterization of liver involvement, a more accurate prognosis, and follow-up^[9]. Under this scenario, accurate identification of the stage of liver fibrosis is critical information in the clinical management of HCC, guiding the choice of therapeutic approach and, mainly, helping to predict the prognosis^[10]. However, it has been challenging. Tests used to stage fibrosis include histological analysis of the liver biopsy and imaging tests.² Liver biopsy is considered the “gold

2

standard” for the diagnosis and staging of liver fibrosis. However, it is an invasive and uncomfortable procedure with a risk of minor (10% to 20%) or more serious (0.5 to 1%) complications. In addition, it is subject to sampling errors and inter-observer subjectivity in the interpretation of histological results [7,11-13]. For staging the grades of fibrosis in biopsied liver tissue, the 0-4 scale of the METAVIR classification system [14] is commonly used and, as noted earlier, the main limitations are related to the representativeness of liver samples and histopathological interpretation. Conventional imaging tests include ultrasonography, computed tomography, and magnetic resonance imaging and, although they represent important tools for detecting cirrhosis, nodules on the liver surface, and splenomegaly, they present low sensitivity for moderate or even advanced fibrosis. Newer acoustic technologies can increase the accuracy of imaging techniques such as hepatic elastography. For these tests, acoustic vibrations are applied to the abdomen and, according to how quickly these vibrations are transmitted along the liver tissue, indicate the stiffness (fibrosis) of the liver. However, some other conditions besides fibrosis also increase liver stiffness [7], which requires further studies and standardizations. Another important limitation is the cost of purchasing the equipment [15], which is unaffordable for places with limited financial resources. In clinical routine, blood markers should be considered a relevant source of information. However, current approaches are limited to combining commonly available tests (e.g., AST, ALT, albumin, serum bilirubin, INR) with clinical information (e.g., age, body mass index, diabetes) and, in some cases, direct markers of liver function. However, this approach is most useful in distinguishing between two levels of fibrosis: absent to minimal *vs* moderate to severe, failing to stratify grades.

It is undeniable that the search for a blood biomarker is a less invasive method of diagnosis/prognosis and as blood circulates through most tissues, it can be a relevant source of information about diseases. Therefore, different molecular biomarkers have been investigated for the characterization of liver fibrosis, especially those with easier and more accessible analytical methodologies [16-20].

In this context, the present study focused on analyzing the plasmatic metabolome of patients affected by chronic hepatitis C with different grades of fibrosis, looking for potential biomarkers to stratify these lesions. Namely, the metabolome is the set of endogenously synthesized metabolites, in a specific physiological condition, and may represent the final product of gene expression, thus, as a secondary aim of this study, we analyzed the pathways linked to the main metabolites detected, adding information about the molecular mechanisms involved in the disease.

MATERIALS AND METHODS

Casuistry

This study was approved by the Ethics Committee on Research of Sao Paulo State University, in conformity with the provisions of the Declaration of Helsinki.

Plasma samples of forty-six volunteer participants diagnosed with hepatitis C (Study Group - HCV) were obtained from peripheral blood. We only included cases of patients older than 18 years, unrelated, diagnosed by detection of HCV-RNA, with identification of HCV genotype, naïve patients (with no previous hepatitis C treatment) and known fibrosis stage or clinical diagnosis of cirrhosis by image. Volunteers with liver transplantation and other liver diseases were excluded. To ensure that the biomarkers found were exclusive to liver lesions (hepatitis C fibrosis), fifty healthy volunteer blood bank donors (Healthy Control Group - CG) were included in the study. Participants included in the study were recruited from the Viral Hepatitis Outpatient Clinic of Botucatu Medical School, UNESP, Brazil. Demographic and clinical characteristics of all study participants, are summarized in Table 1.

Fibrosis was classified using the METAVIR score ^[14]. Liver samples were collected by percutaneous biopsy before any treatment and then analyzed histologically. Peripheral blood was collected at the same time as the liver biopsy.

Sample preparation

Samples were collected in tubes with EDTA anticoagulant, followed by centrifugation to separate the plasma, which was stored at -80°C until the time of metabolite extraction. At the time of extraction, 20 µL of blood plasma was solubilized in 200 µL of tetrahydrofuran (THF), vortexed, and centrifuged at 3,200 rpm for 5 minutes. Then, the collected supernatant was solubilized in 780 µL of methanol and again centrifuged at the same parameters as above. Afterward, 50 µL of this supernatant was solubilized in 500 µL methanol q.s., homogenized, and subjected to chemical ionization with 0.1% formic acid.

Mass spectrometry analysis

For mass spectrometry analysis, the ionized solution was directly injected into an LTQ Mass Spectrometer (ESI-LTQ-XL Discovery, Thermo Scientific, Bremen, Germany) using Electrospray ionization. Ten analytical replicates were performed for each biological replicate. The parameters for analysis were set as the following configuration: sample flow rate of 10 µL/min, capillary temperature of 180°C, 7 kV spray voltage, and carrier gas of 2 arbitrary units. After direct injection, the samples were analyzed in positive ion mode in the mass range of 100-1400 (mass-to-charge ratio) and the signal intensity was detected, which resulted in a set of ions m/z for each sample. The XCalibur software (v. 2.4, Thermo Scientific, San Jose, CA) was used for the acquisition and processing of the data in the spectrometer, which were submitted for statistical analysis.

Statistical analyses

Statistical analysis was performed on the MetaboAnalyst 4.0 platform [21], in which the raw data were evaluated using partial least squares discriminant analysis (PLS-DA). As a result, a list of markers was generated according to the intensity of the most differential and important markers for each group evaluated, i.e., the importance score of each variable in the projection (VIP Score) was obtained. From there, the 6 ions with the highest VIP Score for each grade of fibrosis, whose score was greater than 2.0, were

selected. The accuracy of the identified biomarkers was assessed by receiver operating characteristic (ROC) curve analysis.

Identification of biomarkers

From the selected biomarkers, a search was performed in the METLIN online metabolomics database (<http://metlin.scripps.edu>) to select molecules compatible with the mass/charge values selected for each fibrosis grade. The molecules of interest were added to a candidate list and subsequently fragmented in silico using the MassFrontier tool (v. 6.0, ThermoScientific, San Jose, CA, USA). After the fragmentation of the molecules in silico, the molecules whose fragments obtained were compatible with those generated experimentally were selected.

RESULTS

4

Selection of biomarkers

Based on partial least squares discriminant analysis (PLS-DA), the ions were grouped according to the signal intensity profile within each staging grade, making it possible to comparatively analyze the separation between fibrosis grades, represented in the PLS-DA Score Plot (Figure 1).

To identify the biomarkers responsible for the separation between the groups (represented in Figure 1), a variable importance score (VIP Score) was used in the projection. This score allows visualizing the relevance of each marker within each grade analyzed, according to the mass/charge ratios of the metabolites [23]. Considering a VIP score > 2.0 (Figure 2), the six (6) most important ions were selected for each group (Table 2).

To ensure that the biomarkers found were exclusive to liver lesions (Chronic Hepatitis C - fibrosis, CHC), fifty healthy volunteer blood bank donors (Healthy Control Group - CG) were included in the study. The plasma samples from the two groups (CHC *versus* CG) were compared and this analysis showed that the fibrosis biomarkers (Table 2)

were not detected in the healthy control group (CG). The PLS-DA and VIP Score graphs comparing the two groups are shown in Figures 3 and 4.

Identification of biomarkers

The most relevant biomarkers, represented by m/z values, were identified according to each fibrosis grade, as displayed in Table 2.

Receiver Operating Characteristic (ROC) curve analysis

The accuracy of the biomarkers was assessed by receiver operating characteristic (ROC) curve analysis of the sets of metabolites identified at each fibrosis grade (Figure 5). ROC curves were used to analyze the sensitivity, specificity, and area under the curve (AUC) of each group of metabolites identified at each grade of fibrosis. The ROC curve of the selected metabolites for F1 (AUC = 0.806) was plotted with a sensitivity of 82% and specificity of 68% and the other selected metabolite groups for F2 (AUC = 0.652), F3 (AUC = 0.807) and F4 (AUC = 0.864) showed sensitivity of 62%, 82% and 83% and specificity of 57%, 74% and 76%, respectively.

DISCUSSION

The metabolome has been analyzed in search of new prognostic and diagnostic biomarkers. Thus, the present study investigated differential metabolites in blood plasma as potential biomarkers of fibrosis stages. Our analysis shows potential biomarkers for each grade of liver fibrosis, which may increase the knowledge about the progression of chronic hepatitis C and highlight some targets for further investigation. The biomarkers identified were able to establish an interesting clustering tendency of patients with the same grade of fibrosis, despite some overlaps. The Score Plot analysis showed more efficiency in discriminating the extreme grades (F1 and F4), with overlap in grades F2 and F3. This result may be related to the analytical bias of histological classification since the formation of groups was based on this criterion (METAVIR) [12,13,14].

To ensure that the biomarkers found were exclusive to liver lesions caused by chronic hepatitis C, we compared them to the plasma of healthy donors (Figures 3 and 4). None of the biomarkers found in patient samples (CHC) were detected in the plasma of healthy controls (CG), which reinforces their potential as biomarkers exclusive to the disease.

The analysis of the accuracy of the most relevant metabolites in each grade showed that the sets associated with grades F1, F3, and F4 are good biomarkers (AUC 0.806, 0.807, and 0.864, respectively; Figure 5) and have good sensitivity and specificity scores. However, metabolites identified at grade F2 were less specific and of poor sensitivity. Such findings could be useful in distinguishing grades F1, F3, and F4, where confusion exists when analyses are based on histology alone. Some serum markers of fibrosis validated in patients with hepatitis C patients and correlated with liver biopsy as a reference standard, showed a mean AUC suitable for clinical practice (>0.80) [23], however, an overlap was also observed between adjacent grades of liver fibrosis, particularly in the lower grade of fibrosis [24].

Although the histological bias, our analysis was able to identify different metabolites from diverse chemical classes, including sterols, fatty acids, lipids, and coenzymes, among others. However, for each grade of fibrosis, a metabolite profile has been identified and, as observed in Figure 2, the relevance of each molecule changes according to the fibrosis grade, and may intensify or decrease during the course of the disease.

Some studies have shown the potential of metabolomics analyses for different scenarios in diverse diseases, especially cancer management [25]. One of the great achievements of metabolomics is the assessment of therapeutic response and tumor progression, as shown by Rattner *et al* (2022), in which serum blood metabolites indicated positive or negative response under chemotherapy, using Gas Chromatography-Mass Spectrometry (GC-MS) technique [26]. In addition, some methods for metabolomics analysis have also shown impressive results, such as Nuclear Magnetic Resonance (NMR) and MultiSegment Injection-Capillary Electrophoresis-Mass Spectrometry (MSI-

CE-MS), which were also used to evaluate metabolome of serum samples from chronic HCV patients with fibrosis in different grades [27]. This work showed some interesting markers for the highest grades of fibrosis which are compatible with our results (elucidated below), such as glycerophospholipids and acyl-carnitine markers. Therefore, using metabolomics approaches for liquid biopsies purposes seems to be promising, such as diagnostic, prognostic, and therapeutic monitoring tools.

Within the context of viral infection, it is known that viruses act in the synthesis of fatty acids, taking benefits from their intermediate products. Through lipid synthesis, HCV alters the expression of genes lipid-related, associated with cholesterol biosynthesis [28, 29]. Interestingly, some of the metabolites found in different grades of fibrosis are related to lipid alterations [30-32].

For grade 1 fibrosis (F1), biomarkers were observed that may be more related to HCV infection than to the development of fibrosis per se, if compared to patients with more advanced fibrosis. Thus, the first molecule identified in F1 belongs to the sterols class, with specific signatures for cholesterol ester (CE) ($m/z=671$ and $m/z=672$). Some studies point out that CE is a critical component of lipoviral particles (LVP), whose synthesis has been linked to HCV infection *in vitro* when the accumulation of cholesterol and triglycerides is observed [29]. In association with our results, we suggest that HCV may modulate the environment promoting higher density and infectivity of viral particles and viral spread in hepatic tissue, which intensifies the infection [28, 33, 34].

Still considering the issue of lipid metabolism and accumulation, it was possible to identify the sphingolipid class in the intermediate grade F2, represented by ceramide ($m/z=673$). It represents a central molecule of sphingolipid metabolism, with antiproliferative functions and pro-apoptotic effects [30]. In the setting of HCV infection, lipid accumulation and consequently ceramides occur and may lead to steatosis [35], which may contribute to the development of liver fibrosis [5, 35, 36].

In addition, a glycerolipid was also identified in F1, specified as diacylglycerol (DG) ($m/z=695$). Recent studies point out that the conversion of DG to phosphatidic acid (PA) (mediated by diacylglycerokinases (DGKs)) results in lysophosphatidic acid (LPA)

production, whose signaling is involved in many chronic inflammatory diseases, including fibrosis and cancer [38, 39]. Therefore, the present study highlights a potential relationship between high levels of DG and less fibrotic state (low grade of fibrosis) when compared to F4, where fibrosis is accentuated.

Another lipid class was identified, glycerophospholipids, observed on the intermediate grade F3 and the advanced grade F4, in which the biomarkers were identified as phosphoethanolamines (PE) ($m/z=731$, $m/z=732$ and $m/z=733$). Some studies suggest that PE gradually increases according to the grade of liver fibrosis, acting as a potential marker in the carcinogenesis process [40, 41]. This finding suggests that these patients diagnosed with intermediate grade fibrosis (F3) could be at the beginning of the carcinogenesis process, but this hypothesis needs to be investigated.

Other biomarkers related to changes in lipid signaling pathways have also been found. One of them is represented by the eicosanoid class ($m/z=369$), identified in F2. This molecule is a biologically active lipid that has several implications in biological processes, being a potent mediator of inflammation in infectious diseases and in HCC [42, 43]. In addition, it has been associated with the staging of liver fibrosis, presenting itself as a potential biomarker [44-46]. Another class of lipid, prenol lipid, was identified for the intermediate grade F3, represented by farnesylcysteine ($m/z=365$). This marker participates in the process of liver carcinogenesis by directly acting on the activity of oncogenic RAS proteins [47, 48]. Thus, these results encourage investigations into the use of this metabolite as a potential biomarker of risk for tumor development.

Different intermediate metabolites of the coenzyme A (CoA) class have also been identified, and they are typically involved in the β -oxidation of medium- and long-chain fatty acids to acyl-CoA's (ACS), a key intermediate in lipid metabolism. Some studies suggest the existence of a disruption in fatty acid lipid metabolic pathways during HCV infection [49, 50]. This process results in the accumulation of acyl-CoA and fatty acid metabolic intermediates, such as the next 3 molecules identified in the present study. The *cis,cis*-3,6-Dodecadienoyl-CoA ($m/z=928$) was identified in the F1 cases of our study. For the intermediate grade F3, the marker S-2-Octenoyl CoA ($m/z=914$) was

found [51, 52], and in the advanced grade (F4), a coenzyme A metabolite ($m/z=1118$) was identified. Since different ACS isoenzymes are expressed in the liver, among which some are overexpressed in activated hepatic stellate cells [51,53], the results of the present study indicate that there is a disruption in lipid metabolism all along the infection, but it is not clear and must be investigated. Considering the presence of acyl-CoA's in three different fibrosis grades, these molecules are not good candidates for classification of fibrosis stages but highlight their importance during the course of chronic hepatitis C.

Another marker involved in β -oxidation was found in patients with advanced fibrosis (F4), represented by malonyl carnitine ($m/z=266$). It is known that tumor requires more energy for cell proliferation, which may lead to dysregulation of energy-supplying metabolic pathways, such as β -oxidation of fatty acids [54, 55]. In the context of HCC, alterations in the metabolism of acylcarnitine are directly related to the worsening of the disease and to alterations of β -oxidation [56], which results in the accumulation of ACS [57], discussed previously. Then, it is plausible to suggest malonyl carnitine as a potential HCC biomarker, but it needs more studies to validate this hypothesis.

Besides lipid biomarkers, the polypeptide Angiotensin III (Ang III) ($m/z=931$) was identified in F1, which according to some studies, exhibits physiologically relevant effects similar to those produced by Angiotensin II (Ang II). In the context of chronic hepatitis C and liver fibrosis, Ang III participates in the increase of collagen production through its interaction with the angiotensin type 2 receptor [58, 59], therefore this pathway may be involved at the beginning of the fibrotic process, once Ang III was identified in F1.

The last two metabolites were identified in the intermediate grades, methyladenosine ($m/z=265$) in F2 and (S)-2,3,4,5-Tetrahydropiperidine-2-carboxylate ($m/z=150$) in F3. Adenosine methylation is known as a post-transcriptional modification of messenger RNAs that affects a variety of biological functions [60-62]. In the HCV infection scenario, methyladenosine may represent an RNA modification, which can enhance the production of infectious particles through interactions with viral proteins [62-64]. Thus, this finding suggests that such modifications are involved with the progression of

infection and liver fibrosis. Finally, (S)-2,3,4,5-Tetrahydropiperidine-2-carboxylate, identified in F3, may be related to the degradation of enzymatically inactive proteins and to viral assembly [65]. Although this study relates amino acid residues to the progression of infection and consequent worsening of fibrosis staging, further studies are necessary to clarify the action of these protein residues in the viral cycle. This study has innovative potential regarding the detection of the markers in plasma, a biological fluid easily accessible.

CONCLUSION

In conclusion, these results suggest that some of the observed biomarkers, once validated, have the potential for application as prognostic biomarkers. In addition, this study suggests that liquid biopsy analyzes of plasma metabolites are a good source of molecular biomarkers capable of stratifying patients with chronic hepatitis C according to their fibrosis grade.

ARTICLE HIGHLIGHTS

Research background

1 Chronic hepatitis C (CHC) is an infectious disease caused by the hepatitis C virus (HCV), leading to liver issues like fibrosis, cirrhosis, cancer, and death. The accurate fibrosis stage identification is crucial for treatment decisions and predicting outcomes. Thus, blood markers are a source of relevant information on the staging of fibrosis, in a less invasive and representative way, compared to percutaneous biopsies.

Research motivation

Currently, approaches to staging fibrosis are invasive, subject to sampling errors and subjectivity between observers. In clinical routine, blood markers should be considered a relevant source of information. However, current approaches are limited to routine biochemical tests associated with clinical information, which is not very informative. Analyses based on liquid biopsy are less invasive, and blood plasma, since it circulates

throughout the body, can provide information on pathologies that have not yet manifested themselves clinically, positively impacting on prognosis.

Research objectives

Analyze the plasmatic metabolome of CHC patients, looking for potential biomarkers to stratify these lesions.

Research methods

Plasma metabolites from hepatitis C patients and 50 healthy volunteer participants were analyzed using the LTQ Mass Spectrometer. The sample and the control group were classified into Fibrosis grades was classified using the METAVIR score. Liver samples were collected by percutaneous biopsy before any treatment and then analyzed histologically. The most relevant metabolites were categorized using the METLIN online metabolomics database. The molecules of interest were added to a list of candidates and subsequently fragmented *in silico* using the MassFrontier tool. Molecules compatible with those generated experimentally were then selected for functional analysis.

Research results

For each degree of fibrosis, six differential metabolites were identified that were able to establish an interesting grouping trend among patients with the same degree of fibrosis.

Research conclusions

The results of this study suggest that liquid biopsy analyzes of plasma metabolites are a good source of molecular biomarkers capable of stratifying patients with chronic hepatitis C according to their fibrosis grade.

Research perspectives

Some of the observed biomarkers, once validated, have the potential for application as prognostic biomarkers. This study has innovative potential regarding the detection of pre-clinical biomarkers in easily accessible plasma using minimally invasive methods.

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