Original paper

Profiling of plasma metabolomics in patients with hepatitis C-related liver cirrhosis and hepatocellular carcinoma

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Abstract

Aim of the study: The diagnosis of hepatocellular carcinoma (HCC) is usually late, due to the lack of early detection of biomarkers for HCC. Metabolomics analysis has emerged as a useful tool for studying human diseases. The objective of the study was to investigate the differences in plasma metabolites between hepatitis C virus (HCV)-induced cirrhosis and HCC.

Material and methods: 22 subjects with HCV-related liver cirrhosis and 22 subjects with HCC were enrolled. Clinical, routine laboratory and imaging studies were done. Gas chromatography/mass spectrometry (GC/MS) was used for metabolomics analysis of patients' plasma samples.

Results: 34 known metabolites were detected, of which five metabolites were identified to have the strongest discriminatory power for separation between HCC and cirrhosis groups: octanoic acid (caprylic acid), decanoic (capric acid), oleic acid, oxalic acid and glycine. These are 3 fatty acids (FA), a dicarboxylic acid and a glucogenic amino acid, respectively. No significant correlation was found between the relative intensities of the five metabolites and any of the patient or tumor characteristics (Child-Turcotte-Pugh (CTP) score, Barcelona Clinic Liver Cancer (BCLC) stage, number of focal lesions and size of largest focal lesion). ROC curve analysis was performed and area under the curve (AUC) was calculated, revealing that oleic acid, octanoic (caprylic) acid and glycine had higher positive predictive value than α-fetoprotein.

Conclusions: The study of metabolomics (particularly involving FA) may help define distinct metabolic patterns to distinguish HCV-induced liver cirrhosis from HCC patients. Future research in this field is still needed, particularly concerning HCC treatment strategies which target fatty acid-related metabolic pathways.

Key words: liver cirrhosis, fatty acids, hepatocellular carcinoma, metabolomics, hepatitis C virus (HCV).

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Introduction

Hepatocellular carcinoma (HCC) is increasing in incidence, representing the fifth and ninth most frequently occurring cancer in men and women, respectively [1]. It is considered to be the second leading cause of cancer related mortality in the world, with more than half a million new cases diagnosed worldwide every year. Cirrhosis due to chronic viral hepatitis B or C is now considered the main risk factor for HCC worldwide [2]. Clinically, most patients rarely have symptoms until the later stages of the disease. Thus, the diagnosis is usually late, which is – in turn – responsible for the high morbidity and mortality rates

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associated with HCC [3]. The only chance of long-term disease-free survival in asymptomatic patients depends on early diagnosis of HCC [4]. Ultrasound imaging and serum α-fetoprotein (AFP) have long been considered to be the classic screening methods for early detection of primary liver cancer. However, around 30% of HCC patients are AFP-negative. Therefore, new screening methods for primary liver cancer are increasingly needed [5, 6].

Metabolomics analysis is a new technology which refers to the scientific study of the small-molecular intermediates and products of metabolism. It is a quantitative measurement of endogenous low molecules, with a relative molecular mass of less than 1000 Daltons, hence identifying the unique chemical patterns produced by specific cellular processes [7, 8]. It is a powerful tool in exploring mechanisms of different diseases, including minimal changes in genes and expression of proteins, which provides ample information on discovery of new biomarkers, disease pathogenesis, diagnosis and personalized treatment [9, 10].

Techniques based on mass spectrometry (MS) such as gas chromatography/mass spectrometry (GC/MS) and liquid chromatography/mass spectrometry (LC/MS) are the most widely used and effective technologies in metabolomics analysis. The former is suitable for analyzing the thermally stable, volatile and gaseous compounds of small molecular mass, while the latter can analyze the more polar compounds with higher relative molecular mass and lower thermal stability [11]. GC/MS is considered to be the gold standard technique in metabolomics [8]. It is a collective system where the volatile and thermally stable compounds are first separated by GC, followed by detection of the eluting compounds by electron-impact mass spectrometers. Human blood is a good source for metabolomics research, as there are large amounts of metabolites in blood. Studies have succeeded in extracting and detecting metabolites from human blood by applying GC/MS [12-14].

The analysis of specific patterns of metabolic alterations associated with HCC can help in providing insight into its etiology and mechanisms. This study aims to compare between the plasma metabolite levels in hepatitis C virus (HCV)-related HCC cases and cirrhotic patients, and to evaluate the capability of candidate metabolites in distinguishing between the two groups.

Material and methods

Study population

Forty-four subjects with HCV-related liver cirrhosis with or without HCC were recruited from the Hepatology Department of the Medical Research Institute Hospital, Alexandria University, Egypt during the period from December 2017 to April 2018. Informed consent was obtained from all participants before the study and it was approved by the local Ethics Committee of the institute in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans.

A complete history and physical examination were performed for all patients, followed by analysis of the biochemical and radiological profile. The subjects were divided into two groups: 22 patients with liver cirrhosis due to HCV infection and 22 patients with HCC complicating HCV-related cirrhosis. All patients had no prior history of receiving antiviral therapy. HCV infection was identified by positive serum anti-HCV antibodies, which was confirmed by a polymerase chain reaction (PCR) test. Patients had negative serum markers of active infection with hepatitis B virus (HBV), human immunodeficiency virus (HIV) and schistosomiasis. Also, patients with a history of alcohol consumption > 30 g/day, autoimmune diseases, malignancies, diabetes mellitus and non-HCV related liver cirrhosis were excluded from the study.

Clinical and radiological evaluation

Liver cirrhosis was diagnosed based on clinical, laboratory and imaging criteria (coarse echo pattern of the liver on ultrasound), with reporting of the presence/absence of portal hypertension and splenomegaly. Ascites was graded as none, mild/moderate or severe. Child-Turcotte-Pugh (CTP) score and class were used for assessing the severity of liver disease [15].

Hepatocellular carcinoma cases were diagnosed according to the guidelines of the American Association for the Study of Liver Disease (AASLD) published in 2011, which comprised the presence of a hepatic focal lesion on ultrasound, verified by either a contrast-enhanced triphasic CT-scan study or dynamic contrast-enhanced MRI that showed characteristic criteria for HCC diagnosis (arterial uptake of contrast material followed by washout) [16]. The Barcelona Clinic Liver Cancer (BCLC) system was applied for staging of HCC cases [17].

Biochemical analysis

After an overnight eight-hour fasting period, 10 ml of whole venous blood samples were withdrawn from each subject. One ml was collected in EDTA tubes for complete blood picture and two ml were collected in citrated plasma tubes for prothrombin time and INR

determination. Four ml serum samples were prepared for routine clinical chemistry (using an Olympus AU400 clinical chemistry analyzer; Beckman Coulter, Inc.), according to the methods recommended by the International Federation of Clinical Chemistry and Laboratory Medicine [18]. Serum levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), bilirubin (total and direct), albumin, alkaline phosphatase, gamma-glutamyl transferase (GGT), blood urea nitrogen (BUN) and creatinine were assessed. In addition, serum AFP level was measured for all patients with cirrhosis and HCC (using the automated IMMULITE 1000 immunoassay analyzer; Siemens Medical Solutions Diagnostics Corporation, Erlangen, Germany). A serum cut-off value equal to or more than 200 ng/ml was considered diagnostic for HCC [19]. Anti-HCV-antibodies were measured by immunoassay technique, and HCV RNA load was quantitatively determined using a real-time PCR system.

The remaining three ml of whole blood were collected in separate EDTA tubes and centrifuged at 2000 rpm for 10 min at 4°C. The plasma was aliquoted into Eppendorf tubes and stored at –80°C for metabolomics measurement.

Metabolomics analysis

Chemicals and reagents: N-methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA) with 1% trimethylchlorosilane (TMCS) of > 99.0% purity, methoxyamine hydrochloride (> 98.0% purity) and pyridine (> 99.8% purity) were commercially obtained from Sigma-Aldrich (St. Louis, MO, USA). High performance liquid chromatography (HPLC) grade methanol was purchased from the Tedia Company (Inc., Fairfield, USA).

Sample preparation: Each 100 µl plasma-sample was thawed at 37°C for 10 min, vortexed and mixed for 15 seconds. 800 µl of methanol, 100 µl of distilled water and 10 µl of "heptadecanoic acid in methanol" (1 mg heptadecanoic acid in 1 ml methanol) were added and mixed. Ten µl per sample were vortex-mixed for one minute, kept on ice for 10 minutes, ultrasonicated at room temperature for 5 minutes, then put again on ice for 10 minutes and centrifuged for 10 min (1200 rpm). The supernatant (200 µl) was transferred into a 5 ml glass centrifugation tube and evaporated to dryness by N_2 gas. Next, 50 µl of methoxyamine/pyridine were taken per sample (15 mg/ml = 0.0015 g methoxyamine hydrochloride in 100 µl of pyridine) to the dry tube, vortexed for 30 seconds and left for 16 hours at room temperature with a glass plug. Finally, 50 µl of MSTFA + 1% TMCS derivatization agent were added to the residue, vortex-mixed for 60 seconds and heated in a water bath at 70°C for one hour with a glass plug. The final solution was taken for GC/MS analysis. All the samples were analyzed by GC/MS at random after being preprocessed [12].

GC/MS analysis: Analysis was performed in the research laboratories of City of Scientific Research and Technology Applications, New Borg El-Arab city, Alexandria, Egypt using a Shimadzu GC-2010 gas chromatography instrument coupled with a Shimadzu QP2010 mass spectrometer (Shimadzu Co., Kyoto, Japan). The capillary column used for all analyses was an Agilent DB-5MS with a deactivated fused silica column (inner diameter: 30 m × 0.25 mm, film thickness: 0.25 µm). The column temperature was initially maintained at 80°C for one minute, programmed to 300°C at a rate of 15°C/min, and then held for one min. Ultra high purity helium (99.9%) was used as a carrier gas with a constant flow rate of 1.45 ml/min. The septum purge was turned on with a flow rate of one ml/min all the time. The injector temperature, the interface temperature and the ion source temperature were set at 250°C, 150°C and 230°C, respectively. Ionization was achieved by a 70 eV electron beam. The mass spectrometer was operated under electron impact (EI) in a full-scan mode over the range from m/z 50 to 800 with a 0.5 second scan velocity, and the detector voltage was 0.96 kV [20].

Data processing and statistical analysis

The identification of compounds from the peaks was based on the interpreted tables of m/z values and normalized migration times, by comparing the mass spectrum with Library Spectra v. 2.0 of the National Institutes of Standards and Technology, Gaithersburg, MD (NIST). The identification of metabolites was established by matching masses (m/z) between the peak's fragmentation pattern and the standard database. Peaks with more than 80% similarity were allocated compound names, while those having less than 80% similarity were listed as unknown metabolites. The chromatograms were subjected to noise reduction before peak area integration. Peaks due to noise, column bleed and MSTFA derivatization procedures were excluded from the data set. Integrated peak areas of multiple derivative peaks which belonged to the same compound were added together and considered as a single compound [21].

SPSS version 20.0 software was used in statistical analysis. The discriminant function analysis test was used to determine which metabolites discriminate between the two groups of the study (HCV cirrho-

sis versus HCC). The discriminant function analysis model was created by a retrospective stepwise discriminant approach using the data set of the patients. Initial classification functions were applied to determine to which group each case most likely belongs. After alignment and normalization of significant GC data, multivariate statistical analyses were conducted to estimate the sensitivity and specificity of each significant variable in predicting HCC at a defined cut-off value. Clinical, routine laboratory and imaging data of patients were expressed as mean ± standard deviation

(SD) or proportions. Comparison between two means was performed using the non-parametric Mann-Whitney U-test for abnormally distributed quantitative variables. Comparison between proportions was determined by the chi square (χ^2) test or Fisher's exact test (FET). Spearman's correlation coefficient (r) was applied to our results. A p-value equal to or less than 0.05 was considered to be statistically significant. ROC curve analysis was performed using the relative intensity values of the identified plasma metabolites and the area under the curve (AUC) was calculated to determine their individual ability in predicting HCC cases among cirrhotic subjects.

Results

Clinical, radiological and routine laboratory evaluation

Males represented 40.9% of patients in the HCC group with a mean age of 60.1 years, versus 63.6% of patients in the cirrhosis group with a mean age of 58.6 years ($p > 0.05$). There was no statistically significant difference between the two groups regarding CTP score and class ($p > 0.05$). Triphasic CT evaluation of HCC patients showed that the majority of patients had 2-3 or > 3 focal lesions on presentation (72.8%), the tumor involved both lobes of the liver in 54.5% of patients, with malignant portal vein thrombosis detected in 31.8%, lymph node involvement in only one patient and extrahepatic spread in none. The mean size of the largest focal lesion was 4.43 ±2.01 cm. BCLC staging of HCC patients revealed that more than half of them were at the end stage of the disease (59.1%), while nearly one third of them (31.8%) were at the early stage (Table 1).

The biochemical analysis revealed that there was a statistically significant increase in the levels of AST, ALT, GGT, INR, AFP and white blood cell count (WBC) in the HCC group compared to the cirrhosis group, while there was no statistically significant difference between the two studied groups regarding other parameters (Table 2). Also, there was no statistically significant correlation between serum level of AFP and any of the patient or tumor characteristics (CTP score, BCLC stage, number of focal lesions and size of largest focal lesion ($p > 0.05$).

Metabolomics analysis

Examples of GC-MS total ion chromatograms (TIC) of plasma samples derived from the studied groups are shown in Fig. 1. Around 61 signals were detected in the samples using mass spectral decon-

Table 1. Clinical and radiological data of the studied groups

Variable	HCC (n = 22) n (%)	Cirrhosis (n = 22) n (%)	P-value
Age (years) (X ±SD)	60.09 ±5.06	58.59 ±7.83	0.455
Male sex	9 (40.9)	14 (63.6)	0.131
Splenomegaly by US	12 (54.5)	20 (90.9)	0.007*
Ascites grade by US			0.060
None	1 (4.5)	7 (31.8)	
Mild-moderate	2 (9.1)	2 (9.1)	
Severe	19 (86.4)	13 (59.1)	
CTP score (X ±SD)	11.18 ±2.04	9.91 ±2.91	0.100
CTP class			0.338
Class A	0 (0.0)	2 (9.1)	
Class B	9 (40.9)	9 (40.9)	
Class C	13 (59.1)	11 (50.0)	
PV thrombosis by CT	7 (31.8)	0 (0.0)	0.004*
Number of FL by CT		–	–
Single	6 (27.3)		
Two/three	8 (36.4)		
More than three	8 (36.4)		
Size of largest FL (cm) (X ±SD)	4.43 ±2.01	–	–
Liver lobes involved		–	–
One lobe	10 (45.5)		
Both lobes	12 (54.5)		
LN involvement by CT	1 (4.5)	–	–
Extrahepatic spread	0 (0.0)	–	–
BCLC stage of HCC		–	–
Very early stage	0 (0.0)		
Early stage	7 (31.8)		
Intermediate stage	2 (9.1)		
End-stage	13 (59.1)		

*Data are expressed as mean (X) ± standard deviation (SD) or as number (n) and percent (%), *Statistically significant at p < 0.05, HCC – hepatocellular carcinoma, CTP – Child-Turcotte-Pugh, US – ultrasound, CT – triphasic CT-scan, PV – portal vein, FL – focal lesions, LN – lymph nodes, BCLC – Barcelona Clinic Liver Cancer.*

Table 2. Routine laboratory parameters of studied groups

Parameter	HCC (*n* = 22)	Cirrhosis (*n* = 22)	*P*-value
ALT (U/l)	89.14 ±86.36	27.59 ±12.71	0.002*
AST (U/l)	154.14 ±112.44	55.59 ±26.66	0.001*
Albumin (g/dl)	2.07 ±0.48	2.32 ±0.43	0.076
Total bilirubin (mg/dl)	6.17 ±5.29	3.80 ±6.44	0.189
Direct bilirubin (mg/dl)	4.02 ±3.98	2.32 ±4.63	0.199
Alkaline phosphatase (U/l)	130.95 ±66.30	93.95 ±72.56	0.085
GGT (U/l)	80.64 ±50.73	49.27 ±47.75	0.041*
BUN (mg/dl)	72.41 ±39.15	56.91 ±35.50	0.176
Creatinine (mg/dl)	1.20 ±0.38	1.33 ±0.54	0.357
AFP	276.21 ±252.92	69.93 ±43.82	0.002*
PT (s)	19.01 ±3.14	17.31 ±4.21	0.137
INR	1.64 ±0.23	1.45 ±0.35	0.038*
Hb (g/dl)	10.49 ±1.43	10.46 ±1.50	0.951
RBC (× 10^6 cells/mm^3)	3.12 ±0.60	3.26 ±0.58	0.416
WBC (× 10^3 cells/mm^3)	12.21 ±8.19	8.18 ±3.59	0.041*
Platelets (× 10^3 cells/mm^3)	117.41 ±46.16	118.50 ±62.83	0.948

*Data are expressed as mean ± standard deviation, *statistically significant at p < 0.05, n – number, HCC – hepatocellular carcinoma, ALT – alanine transaminase, AST – aspartate transaminase, GGT – gamma-glutamyl transpeptidase, BUN – blood urea nitrogen, AFP – alpha-fetoprotein, PT – prothrombin time, INR – international normalized ratio, Hb – hemoglobin, RBC – red blood cell count, WBC – white blood cell count.*

volution software for peak detection. However, many of them were not consistently found in other samples or presented too low abundance or too poor spectral quality to be accurately assigned to specific metabolites. A total of 34 peaks could be auto-identified by the NIST library through comparing the fragmentation patterns composed of all fragment ions, as shown in Table 3. The remaining peaks which could not be identified were not reported. These metabolites are suggested to be involved in energy metabolism, lipid metabolism, protein metabolism, and amino acid metabolism.

Five metabolites were finally selected among all variables with the strongest discriminatory power for separation between the HCC group and the liver cirrhosis group. The five peaks were identified as octanoic acid (caprylic acid), decanoic (capric acid), oleic acid, oxalic acid and glycine, which were all significantly higher in the HCC patients compared to the HCV-cirrhotic group.

When statistically comparing the mean values of the relative intensities of the five plasma metabolites between the two studied groups, they were found to be significantly higher among the HCC group compared to the cirrhosis group (Table 4). However, no statistically significant correlation was found between the relative intensities of the five metabolites and any of the patient or tumor characteristics (CTP score, BCLC stage, number of focal lesions and size of largest focal lesion), as shown in Table 5.

ROC curve analysis was performed using the relative intensity values of the identified plasma metabolites in comparison to serum concentration of AFP. The area under the curve (AUC) was calculated to determine their individual ability in predicting HCC cases among cirrhotic subjects, revealing that oleic acid, octanoic (caprylic) acid and glycine had higher positive predictive value than AFP (Table 6, Fig. 2).

Discussion

Hepatocellular carcinoma is often advanced and incurable at presentation, which is partially attributed to the absence of appropriate biomarkers for early diagnosis [22]. The technology of metabolomics has emerged as a useful analytical tool for human disease study, because of its high sensitivity and capability to simultaneously measure many metabolites [23].

The objective of the present study was to investigate the unique differences in plasma metabolites between HCV-cirrhotic and HCC patients. Based on non-targeted metabolomic analysis, the present work detected 61 metabolites, of which 34 metabolites were known
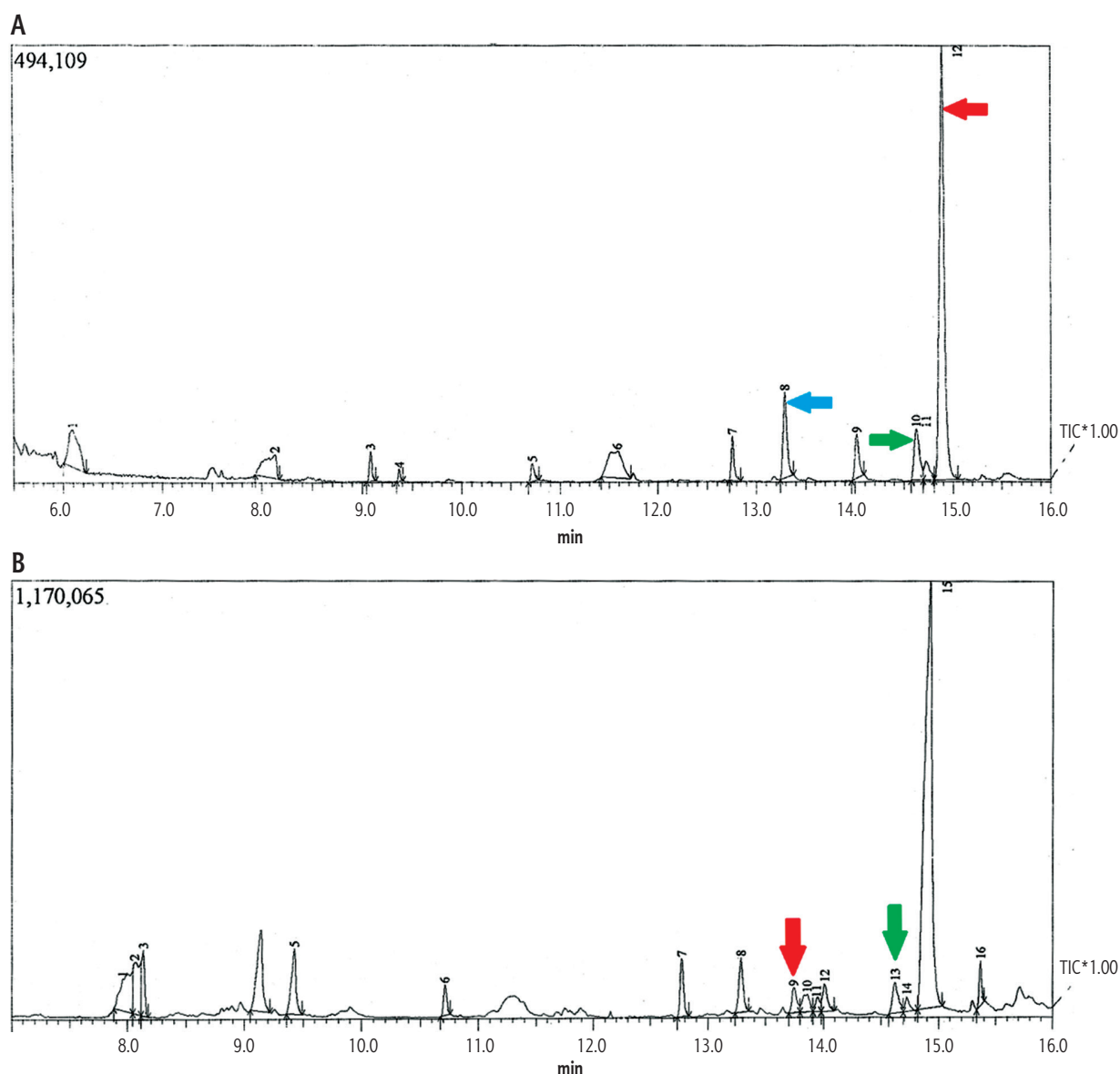
Fig. 1. Example of GC-MS total ion chromatogram (TIC) of plasma sample of HCC versus cirrhosis patient. **A**) HCC patient chromatogram demonstrating high peaks of decanoic acid (blue arrow), oleic acid (green arrow), and glycine (red arrow); **B**) Cirrhosis patient chromatogram demonstrating low peaks of glycine (red arrow) and oleic acid (green arrow), while decanoic acid was undetectable

compounds. Five metabolites were successfully identified with the strongest discriminatory power for distinguishing between the HCC group and the liver cirrhosis group, namely octanoic acid (caprylic acid), decanoic (capric acid), oleic acid, oxalic acid and glycine. The first two of the identified metabolites are medium-chain saturated FA, oleic acid is a monounsaturated FA, oxalic acid is a saturated dicarboxylic acid, while glycine is a simple glucogenic amino acid. ROC curve analysis demonstrated that oleic acid, octanoic (caprylic) acid and glycine had higher ability than AFP in predicting HCC cases among HCV-cirrhotic patients.

Hepatocellular carcinoma, as well as other malignant tumors, is known to generate a catabolic state in the body. The liver, being the principal metabolic hub of fats, carbohydrates and proteins, undergoes major metabolic alterations under these circumstances. HCC malignant cells require building blocks to provide material for cellular membranes, signaling molecules and energy as they proliferate and spread. Glycolysis, of course, acts as the primary source of energy [24]. In addition, gluconeogenesis from lipids and proteins also plays a key role, which consequently increases the turnover of glucogenic amino acids (e.g. glycine, alanine) and free FA [25, 26]. This might be one explanation for the findings of our metabolomics analysis. In fact, a metabolomics study by Di Poto *et al.* identified glycine as having better performance than AFP, and

Table 3. Discriminant analysis of different plasma metabolites in the studied groups

No.	Identified metabolites	Retention time (min)	m/z ratio	HCC $n = 22$	Cirrhosis $n = 22$	Wilks' lambda	P-value
1	Dihydroxyacetophenone	5.793	281.00	5.132 ±3.905	5.475 ±1.021	0.971	0.078
2	Trisiloxane	6.136	73.00	8.220 ±4.657	7.545 ±4.232	0.949	0.084
3	Benzenedicarboxaldehyde	6.958	133.00	2.733 ±1.942	1.043 ±0.986	0.983	0.400
4	Carbamic acid	7.304	149.00	7.645 ±7.578	2.817 ±1.910	0.917	0.058
5	3-ethyl 2-methylhexane	7.465	84.00	5.857 ±1.443	4.675 ±0.007	0.940	0.069
6	Silane	7.585	207.00	2.860 ±0.000	1.193 ±0.543	0.995	0.635
7	Pyridinecarbonitrile	7.811	221.00	5.66 ±0.000	14.120 ±0.000	0.994	0.605
8	Caprylic acid	8.106	57.00	7.334 ±2.623	2.711 ±1.802	0.985	0.023*
9	Oxomalonic acid	8.087	57.00	3.770 ±0.000	2.736 ±2.006	0.988	0.480
10	Oxalic acid	8.110	57.00	3.223 ±1.001	2.112 ±0.701	0.934	0.001*
11	Neohexane	9.050	57.00	3.950 ±0.000	1.090 ±0.000	0.952	0.155
12	Enanthic acid	9.078	57.00	2.945 ±0.306	3.983 ±2.448	1.000	0.985
13	Caproic acid	9.084	57.00	2.945 ±0.306	3.983 ±2.448	1.000	0.985
14	Butane	9.315	57.00	2.731 ±1.228	4.770 ±0.000	0.976	0.319
15	Iodododecane	9.386	57.00	8.570 ±0.000	2.060 ±0.000	0.949	0.141
16	Valeric acid	9.629	191.00	3.3120 ±2.000	3.146 ±2.153	0.997	0.704
17	Glutaric acid	9.697	191.00	3.312 ±2.000	3.146 ±2.153	0.997	0.704
18	Methoxy benzoic acid	10.617	135.00	3.801 ±2.025	2.564 ±1.593	0.961	0.199
19	Ethanol	12.668	179.00	5.025 ±2.147	3.634 ±2.023	0.973	0.290
20	Hypoxanthine	13.115	55.00	1.800 ±0.000	0.650 ±0.000	0.982	0.389
21	Arachidic acid	13.133	73.00	3.129 ±2.491	3.424 ±5.271	0.991	0.535
22	Palmitic acid	13.221	73.00	3.129 ±2.491	3.424 ±5.271	0.991	0.535
23	Pentadecylic acid	13.234	73.00	3.129 ±2.491	3.424 ±5.271	0.991	0.535
24	Heptadecanoic acid	13.893	57.00	1.920 ±1.392	1.983 ±1.352	0.988	0.484
25	Propionic acid	14.547	57.00	1.382 ±1.268	0.616 ±0.220	0.929	0.081
26	Capric acid	14.601	73.00	0.713 ±0.210	0.343 ±0.208	0.923	0.009*
27	Oleic acid	14.621	55.00	4.743 ±1.442	1.602 ±0.333	0.929	0.041*
28	Stearic acid	14.747	60.00	4.460 ±0.057	2.440 ±2.258	0.997	0.721
29	Glycine	14.774	223.00	55.245 ±1.734	50.611 ±2.341	0.939	0.015*
30	Methionine	15.153	223.00	1.071 ±0.663	0.947 ±0.658	0.995	0.639
31	L-Leucine	15.193	223.00	1.071 ±0.663	0.947 ±0.658	0.995	0.639
32	Butylhydroquinone	15.273	295.00	1.737 ±0.313	1.525 ±0.335	0.994	0.102
33	Acrylic acid	16.164	311.00	14.200 ±0.000	6.070 ±6.772	0.996	0.674
34	Isophthalic acid	16.904	267.00	57.710 ±0.000	50.360 ±19.638	0.976	0.318

*Relative intensities of plasma metabolites in the two study groups (HCC vs. cirrhosis) are given as their peak areas, and expressed as mean ± standard deviation, *Statistically significant at p ≤ 0.05, n – number, HCC – hepatocellular carcinoma.*

oxalic acid as clearly distinguishing HCC cases from HCV-cirrhotic controls [27]. Another metabolomics study by Muir *et al.* demonstrated elevated oleic, adrenic, and osbond acids in the plasma of patients with nonalcoholic steatohepatitis-associated hepatocellular carcinoma [28]. Furthermore, a third study by Qiu *et al.* used chromatography–mass spectrometry to prove that linoleic acid, oleic acid, arachidonic acid and palmitic acid were potential fatty acid biomarkers of HCC patients [29].

Cancer-induced dysregulation of FA metabolism has been receiving particular attention in recent years.

Table 4. Comparison between the two studied groups according to the relative intensities of the five identified plasma metabolites

	HCC group (n = 22)	Cirrhosis group (n = 22)	Test of sig.	P-value
Octanoic (caprylic) acid				
Range	3.5-11.2	0.9-6.4	U = 9.0*	< 0.001*
Mean ±SD	7.3 ±2.6	2.7 ±1.8		
Oxalic acid				
Range	1.6-4.7	1-3.1	t = 2.774*	0.011*
Mean ±SD	3.2 ±1.1	2.1 ±0.7		
Decanoic (capric) acid				
Range	0.3-1.1	0-0.7	U = 22.0*	0.003*
Mean ±SD	0.7 ±0.2	0.3 ±0.2		
Oleic acid				
Range	2.6-6.9	1-2.1	t = 7.053*	< 0.001*
Mean ±SD	4.7 ±1.4	1.6 ±0.3		
Glycine				
Range	52.1-57.3	45.8-53.6	t = 5.369*	< 0.001*
Mean ±SD	55.2 ±1.7	50.6 ±2.3		

U – Mann-Whitney test, t – Student's t-test, p – p value for comparison between the two studied groups, *statistically significant at p < 0.05

It was suggested that cancer cells fulfill their requirement for energy and building materials either by up-regulating *de novo* FA synthesis, or by altering FA oxidation [30]. At this point, studies seem to disagree on how FA regulation is involved in tumorigenesis. While some studies linked the downregulation of FA oxidation with HCC [31], others associated increased catabolism of certain saturated lipids with high AFP levels in the serum of HCC patients, concluding that lipidomics analysis may provide new biomarkers for HCC [32-34]. Li *et al.* also demonstrated that aberrant lipid metabolism was an evident feature of HCC, and that the severity of the condition correlated with higher tissue concentrations of saturated triglycerides (TG) and lower concentrations of polyunsaturated TG [35]. Lin *et al.* revealed similar outcomes and concluded that their findings offer the biomedical potential to use the altered lipid metabolism as a diagnostic marker for cancer cells, which – in turn – opens the opportunity for treating aggressive HCC by targeting altered lipid metabolism pathways [36].

Nevertheless, our results showed no correlation between patient/tumor characteristics (CTP score, BCLC stage, number of focal lesions and size of largest focal lesion) and the relative intensities of the identified

Table 5. Relation between relative intensities of the five plasma metabolites and different patient and tumor characteristics

	Octanoic (caprylic) acid	Decanoic (capric) acid	Oleic acid	Oxalic acid	Glycine
CTP-score (n = 44)	r = –0.131 p = 0.542	r = 0.171 p = 0.426	r = 0.244 p = 0.251	r = 0.246 p = 0.256	r = 0.182 p = 0.395
BCLC stage* (n = 20)	U = 9.0 p = 0.833	U = 8.0 p = 0.667	t = 1.351 p = 0.214	t = 0.389 p = 0.707	t = 0.572 p = 0.583
Number of FL (n = 22)	H = 0.932 p = 0.628	H = 1.682 p = 0.431	F = 0.332 p = 0.727	F = 1.223 p = 0.344	F = 1.448 p = 0.291
Size of largest FL (n = 22)	r = 0.124 p = 0.717	r = 0.417 p = 0.202	r = 0.096 p = 0.778	r = 0.272 p = 0.418	r = 0.453 p = 0.161

CTP – Child-Turcotte-Pugh, BCLC – Barcelona Clinic Liver Cancer (*Intermediate stage patients were excluded from analysis due to small sample size, n = 2), FL – focal lesion, n = number of patients, r – Pearson coefficient, U – Mann-Whitney test, t – Student's t-test, F – ANOVA test, H – Kruskal Wallis test, p – level of significance between the different categories (statistically significant at p ≤ 0.05)

Table 6. Sensitivity and specificity of AFP versus plasma metabolites in predicting HCC cases among cirrhotic patients

	AUC	P	95% CI	Cut-off	Sensitivity	Specificity	PPV	NPV
AFP	0.867*	0.002*	0.725-1.009	> 95	72.73	84.62	80.0	78.6
Octanoic (caprylic) acid	0.937*	< 0.001*	0.847-1.028	> 5.003	81.82	92.31	90.0	85.7
Oxalic acid	0.762*	0.030*	0.552-0.973	> 3.142	63.64	100.0	100.0	76.5
Decanoic (capric) acid	0.846*	0.004*	0.691-1.001	> 0.512	72.73	84.62	80.0	78.6
Oleic acid	1.000*	< 0.001*	1.000-1.000	> 2.087	100.0	100.0	100.0	100.0
Glycine	0.951*	< 0.001*	0.871-1.031	> 53.57	81.82	100.0	100.0	86.7

AUC – area under curve, p – value: probability value, CI – confidence interval, NPV – negative predictive value, PPV – positive predictive value, AFP – alpha-fetoprotein, *statistically significant at p ≤ 0.05

plasma metabolites. In comparison, Muir *et al.* found a great discrepancy among the different identified fatty acids and their relation to HCC tumor size/burden in mice and humans. While some of them showed a positive correlation (e.g. oleic and adrenic acids), others showed negative (e.g. margaric and linoleic acids) or no correlation with tumor size/burden [28].

In Egypt, cases of hepatocellular carcinoma are mostly secondary to HCV-induced liver cirrhosis [37]. HCV infection seems to have a synergistic effect on lipid turnover, namely by encouraging lipogenesis and steatosis to provide a lipid-rich environment for viral replication [38]. This is achieved by augmenting the expression and activation of specific transcription factors that activate the synthesis of FA, triglycerides and cholesterol, causing their accumulation in the liver [39]. Added to the previously described HCC-related alteration of lipid metabolism, these findings emphasize the role of dysregulated FA particularly in HCV-induced HCC carcinogenesis, and indicates that interfering with lipogenesis may represent a potential therapeutic strategy for these cases.

Numerous therapeutic agents are already targeting key and/or lipogenic enzymes and pathways in lipid metabolism and have shown good efficacy against several cancers. However, this progress in therapeutic agents for HCC seems to be lagging behind. This might be explained by the great genetic and biochemical variability among HCC patients, which makes it difficult to classify them based on lipid metabolism. A second explanation is the complexity of FA metabolism itself, which involves an active balance between synthesis and catabolism [40].

Conclusions

From this perspective, we conclude that the study of metabolomics may help define distinct metabolic patterns, particularly in FA metabolism, which may distinguish HCV-induced liver cirrhosis from HCC patients, hence aiding in early diagnosis of this fatal condition. Future research in this field is still needed, particularly concerning HCC treatment strategies which target fatty acid-related metabolic pathways.
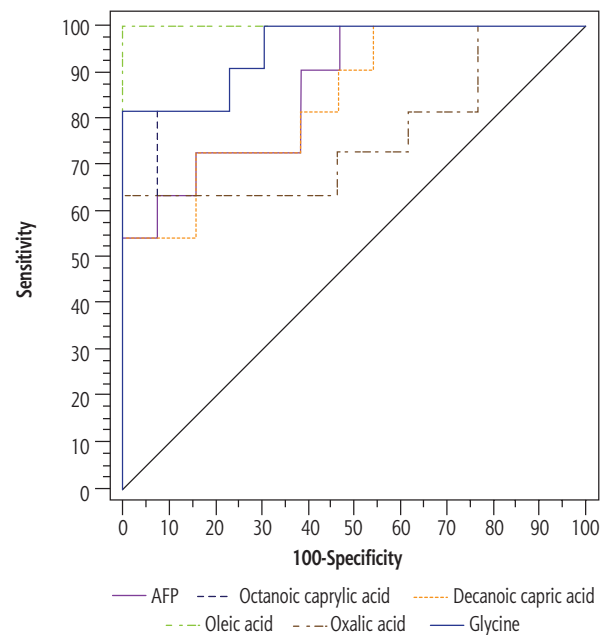
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Fig. 2. ROC curves of AFP and plasma metabolites demonstrating their different abilities to predict HCC cases among cirrhotic patients

Disclosure

The authors report no conflict of interest.

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